# The influence of biologic therapy on the capacity of regulatory T cells to restrain Th17 responses

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## Declaration:

I, Jenny McGovern confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

## Abstract

The importance of IL-17 is underscored both by its resistance to control by regulatory T cells (Treg) and the propensity of Treg to produce this highly inflammatory cytokine. I addressed whether Th17 cells are inhibited by Treg from anti-TNF treated rheumatoid arthritis (RA) and psoriatic arthritis patients (PsA) and defined the underlying mechanisms.

Th17 responses were inhibited by Treg isolated from RA patients responding to the anti-TNF antibody adalimumab, but not by Treg from healthy individuals or patients with active RA. Furthermore, in patients with RA, response to adalimumab therapy was associated with a reduction in $RORC^+$ Th17 cells and an increase in $FOXP3^+$ Treg lacking Helios and CD62L expression. These Treg suppressed Th17 cells through inhibition of monocyte-derived IL-6, but independently of IL-10 and TGF-ß, which mediated suppression of Th1 responses. Surprisingly, therapy with the anti-IL-6 receptor, tocilizumab, did not result in a reduction in $RORC^+$ cells in RA patients. Rather, tocilizumab reduced T cell IL-21 production, which was associated with a diminished memory B cell population.

The acquisition of IL-17 suppressor function by Treg was not observed in RA patients responding to etanercept, a modified TNF receptor, or in PsA patients treated with either adalimumab or etanercept. Moreover, response to therapy was not associated with an increase in Treg number in these patients. In RA patients treated with etanercept the inability of Treg to suppress Th17 responses was associated with high levels of IL-17 production and high levels of $RORC^+$ Th17 cells *ex vivo*. In

contrast, there was a reduction in IL-17 production and $RORC^+$ Th17 cells *ex vivo* in PsA patients treated with both adalimumab and etanercept. Furthermore, depletion of Treg from PBMC showed that Treg from healthy controls, patients with active PsA and PsA patients responding to adalimumab can modulate the production of IL-22, a key cytokine in inflammatory skin disorders. However, PsA patients responding to etanercept have an impaired ability to regulate production of this cytokine.

In conclusion, the induction of IL-17 suppressing Treg by anti-TNF is both therapy and disease specific. These data provides a rationale for the therapeutic benefit of switching between different anti-TNF agents. Furthermore, the induction of highly potent Treg may offer an explanation as to why patients treated with adalimumab have an increased risk of developing *Mycobacterium tuberculosis* (TB).

## Accepted abstracts and publications

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## Abbreviations

Nrp-1 - neuropilin-1

7AAD - 7-Aminoactinomycin D

ACPA - anti-citrullinated protein antibodies

NR - non-responder

AdTreg - Treg from patients treated with adalimumab

AhR - aryl hydrocarbon receptor

AIA - antigen-induced arthritis

APC - antigen presenting cells

AS - ankylosing spondylitis

ATP - adenosine triphosphate

BSA - bovine serum albumin

CD – cluster of differentiation

CIA - collagen induced arthritis

CNS - conserved non-coding DNA sequences

CSF - colony stimulating factors

CREB - cAMP response element-binding

CTLA-4 - cytotoxic T-lymphocyte antigen 4

CV - cardiovascular disease

DAS-28 - disease activity score - 28

DC - dendritic cells

DD - death domains

DMARDS - disease modifying anti-rheumatic drugs

EAE - experimental autoimmune encephalomyelitis

EBI3 - Epstein-Barr virus induced gene 3

ELISA - enzyme-linked immunosorbent assay

EtTreg - Treg from patients treated with etanercept

FACS - fluorescent activated cell sorting

FCS - fetal calf serum

FLS - fibroblast-like synovial cells

GARP - glycoprotein A repetitions predominant

GC - germinal centre

GITR - glucocorticoid-induced tumour-necrosis factor receptor-related protein

GM-CSF - granulocyte-macrophage colony-stimulating factor

GWAS - genome-wide association studies

*Gzma* - granzyme A

HIF-1 - hypoxia inducible factor 1

HLA - human leukocyte antigen

HTreg - Treg from healthy controls

ICAM - intercellular adhesion molecule

IDO - indoleamine 2,3-dioxygenase

IFNγ - interferon gamma

IL - interleukin

IL-6R - IL-6 receptor

IPEX - immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome

iTr35 - Treg induced by IL-35

iTreg - induced Treg

IκBα kinase - IKK

LAG-3 - lymphocyte-activation gene 3

LPS - lipopolysaccharide

LT-β - lymphotoxin beta

mAb - monoclonal antibody

mBSA - methylated bovine serum albumin

MFI – mean fluorescence intensity

MHC - major histocompatability complex

MMPs - matrix metalloproteinases

mTNF - membrane bound TNF

MTX - methotrexate

NFAT - nuclear factor of activated T-cells

NFκB - nuclear factor kappa-light-chain-enhancer of activated B cells

NICE - National Institute for Health and Clinical Excellence

nTreg - natural Treg

OPG - osteoprotegerin

OVA - ovalbumin

PASI - psoriasis area severity score index

PBMC - peripheral blood mononuclear cells

PBS – phospate buffered saline

PD-1 - programmed death 1

PsA - psoriatic arthritis

PsARC - psoriatic arthritis response criteria

PTPN22 - protein tyrosine phosphatase, non-receptor type 22

RA - rheumatoid arthritis

RANK - receptor activator of nuclear factor kappa-B

RATreg – Treg from patients with active RA

RF - rheumatoid factor

ROI - reactive oxygen intermediates

ROR - retinoic-acid-receptor-related orphan receptors

Runx-1 - runt-related transcription factor 1

SE - Standard Error

SNPs - single nucleotide polymorphisms

STAT - signal transducer and activator of transcription

sTNF - soluble TNF

TB - *Mycobacterium tuberculosis*

TCDD - 2,3,7,8-tetrachlorodibenzo-p-dioxin

TCR - T cell receptor

TGF-β - transforming growth factor beta

Th – T helper

TIMPs - tissue inhibitors of metalloproteinases

TLR - toll-like receptors

TMB - tetramethylbenzidine

TNF – tumour necrosis factor

TNFR - TNF receptor

TNIP1 - TNFAIP3-interacting protein 1

Treg - regulatory T cell

Tresp - responder T cell

VCAM - vascular cell adhesion molecule

VEGF - vascular endothelial growth factor

αCD3/28 - anti-CD3 and anti-CD28

αCD39 - anti-CD39

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# Chapter 1

# Introduction

## 1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease which affects approximately 1% of the population, with 3 females affected for each male [1]. It is a systemic disorder affecting peripheral joints such as hands, wrists, feet and knees in a symmetrical fashion. Disease is associated with severe deformity and in the early 1990s was estimated to cost £1.256 billion per year in England, with 52% of this sum as a result of work-related production loss through disability [2].

In addition to severe disability, it has been known since the 1950s that RA is associated with increased mortality [3] both from the disease itself but also from associated co-morbidities. A number of factors have been found to correlate with premature mortality in RA; these include the presence of rheumatoid factor and rheumatoid nodules at first visit and long-term disease severity [4-6]. Moreover, RA patients have increased mortality from infections, respiratory disease, non-Hodgkin's lymphoma and cardiovascular disease (CVD) than the general population [6, 7]. Indeed the mortality rate from CVD in patients with RA is 50-100% higher than the general population and has been shown to be predicted by baseline C-reactive protein (CRP) levels [8] and persistently high disease activity [9].

Classification of RA is based on 4 main criteria: the numbers of swollen joints, the presence of autoantibodies, the presence of acute phase proteins and the duration of symptoms. Patients receive a score for each of these criteria and are classified as RA if their score is $\geq$ 6/10 [10].

Disease activity is measured by the disease activity score (DAS)-28. DAS-28 score is calculated based on the tenderness and swelling in 28 joints, erythrocyte sedimentation rate (ESR) and a global health assessment. Joint damage is measured by assessing joint space narrowing and bone erosions on radiographs.

### 1.1.1 The immunopathogenesis of rheumatoid arthritis

In a healthy individual tolerance to self-tissue is maintained through the removal of potentially pathogenic cells during development in the thymus, a process named central tolerance. If pathogenic cells should escape deletion and leave the thymus there are mechanisms in place to control these cells in the periphery, known as peripheral tolerance. In order for an autoimmune disease such as RA to develop, tolerance to self-tissue must be breached. There are a number of factors that contribute to the breach of tolerance in RA and these factors may differ between individuals. There is substantial evidence for a genetic association with RA, with twin studies showing approximately 65% heritability [11]. Genetic similarities in RA-susceptible individuals are commonly mutations in a single base pair of genes, referred to as single nucleotide polymorphisms (SNPs). Many of the SNPs associated with the development of RA are important for key immune responses. However, RA is not 100% heritable, which means there must be non-genetic factors, which

contribute to the development of disease. These non-genetic elements include exposure to toxic substances such as cigarette smoke, infection, trauma and hormonal factors. A breach of peripheral tolerance leads to the inappropriate presentation of joint specific antigens to T cells in the synovium and the subsequent chronic inflammation that fails to be suppressed by natural mechanisms.

The pathogenesis of RA can be divided into 5 stages (Table 1.1, pg 27). Each of these stages is associated with distinct immunopathology. In the text that follows the contributing factors to the immunopathogenesis of RA are discussed to give a picture of how tolerance is breached in RA and how this leads to chronic inflammation and joint destruction. Moreover, the latter part of this text discusses how RA pathogenesis and joint destruction can be targeted for the amelioration of disease.

**Table 1.1. Immunopathogenesis of rheumatoid arthritis.**

| Stage | Immunology | Clinical Symptoms |
|---|---|---|
| 1.<br><br><br>*Breach of tolerance* | Central tolerance<br>Peripheral tolerance<br>Genetic susceptibility<br>Environmental factors (necrosis and citrullination of proteins associated with smoking).<br>Infection (molecular mimicry)<br>Trauma (joint damage targets already primed immune system to the joint).<br>Hormones (oestrogen drives IL-1)<br><br><br>Presentation of self-antigen to T cells<br>Autoantibody production. | Few clinical symptoms, though antibodies against citrullinated proteins are known to be present up to 10 years before onset of disease [12, 13]. |
| 2. | Lymphocyte proliferation and differentiation of Th17 and Th1 cells<br>Angiogenesis of the synovial membrane.<br>Granulocyte recruitment to the joint. | Characterised by pain and swelling of the small joints of the hands and feet. Mild joint stiffness. |
| 3.<br><br>*Inflammation* | Further recruitment and accumulation of neutrophils – leading to further production of chemokines such as CCL2 and CXCL8.<br>Recruitment of monocytes and conversion to macrophages – antigen presentation and production of TNFα and IL-1 to activate cells of the synovium and enhance cellular recruitment.<br>Accumulation of functional regulatory T cells, but no impairment of inflammation.<br>Synovial cell hyperplasia. | The knees, ankles, hips, and the cervical spine and lower jaw become tender and swollen; this is accompanied by large inflammatory nodular masses within these joints. Warm swollen joints, pain, and limited motion. |
| 4.<br><br>*Joint Damage*<br><br>↓ | Formation of a pannus containing inflammatory cells producing cartilage-degrading enzymes.<br>Activated osteoclasts, chondrocytes and synovial fibroblast-like cells contribute to destruction of cartilage.<br>Synovial membrane takes on anchorage independent phenotype with altered oncogene expression and reduced cell death. | As above with increased swelling, radiographic evidence of osteopaenia. |
| 5. | Pannus invasion, destruction of cartilage and subchondral bone.<br>Chondrocyte proliferation. | Loss of function and deformity, narrowing of joint spaces and increased extraarticular complications. |

Adapted from [14]

#### *1.1.1.1 Central Tolerance:*

Mature T cells are characterised by their surface expression of a heterodimeric T cell receptor (TCR) made up of an alpha chain and a beta chain, which recognises peptide- major histocompatability complex (MHC). The TCR is associated with a second cell surface receptor cluster of differentiation (CD) 3, important for stabilising the receptor complex at the cell surface and for downstream signalling [15]. In addition either CD4 or CD8 are expressed and these receptors recognise MHC class II or MHC class I, respectively. It is now known that T cells and B cells develop from the same progenitor stem cell in the bone marrow, but T cells traffic to the thymus to complete the maturation process. In order to generate T cells with a wide range of specificities, random rearrangement of T cell receptors (TCR) takes place during development. In the thymus, cells progress through a series of checkpoints to test the specificity of these randomly generated TCR. This results in the production of mature cells with high specificity for foreign antigens and low specificity for tissue proteins (self-antigen).

The first checkpoint is named positive selection. During this stage, developing T cells that fail to recognise autologous MHC molecules die by neglect. At the second checkpoint, an intracellular protein named AIRE drives the ectopic presentation of peripheral tissue proteins on MHC in the thymic epithelium [16]. If the TCR has a high affinity for the antigen expressed, a strong signal will be transduced and the cell will undergo clonal deletion in a process named negative selection [17]. Alternatively, if the TCR has a low affinity for the antigen presented it will survive and proceed to enter the circulation.

The importance of TCR signalling for the selection of the peripheral T cell pool is highlighted in elegant experiments where CD3ζ-deficient mice in which TCR signalling is ablated were crossed to mice in which all T cells possess a TCR for a male antigen, HY. In the absence of a TCR signal, male mice failed to negatively select auto-reactive HY specific T cells resulting in an autoimmune pathology. In female mice however, the absence of both antigen expression in the tissues and a TCR signal resulted in the complete ablation of T cells in the periphery [18], suggesting that developing thymocytes require a TCR signal. Furthermore it has been shown that SKG mice that spontaneously develop autoimmune arthritis do so due to a mutation in ZAP-70, an important molecule in the proximal stages of TCR signalling. Autoimmunity in these mice was shown to be driven by a reduction in TCR signalling leading to the positive selection of auto-reactive T cells [19]. Thus a T cell signal is vital to survival of developing thymocytes, but a strong signal through the TCR leads to T cell deletion in order to prevent potentially self-reactive cells causing harm in the periphery.

#### *1.1.1.2 Peripheral Tolerance*

T cells are selected on a gradient of reactivity and so some cells that enter the circulation will be more strongly self-reactive than others. Moreover, not all autologous proteins can be expressed in the thymus. Thus, there must be mechanisms in place to suppress immune response to self-antigens in the peripheral tissues. These include anatomical sequestration, the induction of anergy and the activity of regulatory T cells (Treg) [20].

*Anatomical sequestration:*

The circulation of naive T cells, mediated by the chemokine receptor CCR7 and its ligands, ensure that cells that have not experienced antigen never enter the non-lymphoid peripheral tissues. This reduces the possibility of self-antigen being presented to T cells.

*Anergy:*

In contrast to their naive counterparts, T cells that have encountered antigen can migrate into the tissues. However, the transfer of T cells with a TCR specific for the protein ovalbumin (OVA) to mice with expression of OVA on peripheral tissues does not result in autoimmunity. Only upon secondary damage to the tissue could a robust OVA-mediated pathology be triggered [21]. Thus, the presence of autoreactive T cells in the tissue is not sufficient to drive autoimmunity.

T cell activation in the periphery requires interaction with antigen presenting cells (APC) expressing an antigenic peptide for which the T cell is specific. Additionally, T cells require a co-stimulatory signal to become activated. The best-characterised co-stimulatory interaction is that of CD28 on T cells and B7 family members (in particular B7-1/CD80 and B7-2/CD86) expressed on APC. CD28 lowers the threshold required for activation of T cells via the TCR [22]. Mice which are deficient in CD28 have impaired T cell response to antigen with a reduced activation of helper T cells and reduced antibody class switching [23]. Thus, binding of B7 family members to CD28 in combination with a TCR signal leads to an increase in

the transcription and production of interleukin (IL)-2 to promote T cell proliferation [24].

If a T cell recognises an antigen but does not receive this second co-stimulatory signal it becomes functionally unresponsive, or anergised [25] (Figure 1.1). In the absence of inflammation APC do not up-regulate co-stimulatory molecules. Cells which recognise antigen in this environment may undergo proliferation but will fail to become pathogenic [20]. T cell anergy can also be induced by interaction of T cells with tolerogenic dendritic cells (DC) which have undergone incomplete maturation due to the uptake of apoptotic cells [20]. Furthermore, recent data suggest that the induction of anergy may not be a passive process, as members of the CD28 family including cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death 1(PD-1) have been shown to negatively regulate T cell co-stimulation [26].

Thus, in order to break tolerance, self-reactive T cells must escape negative selection in the thymus. These cells must then encounter antigen in the context of inflammation.

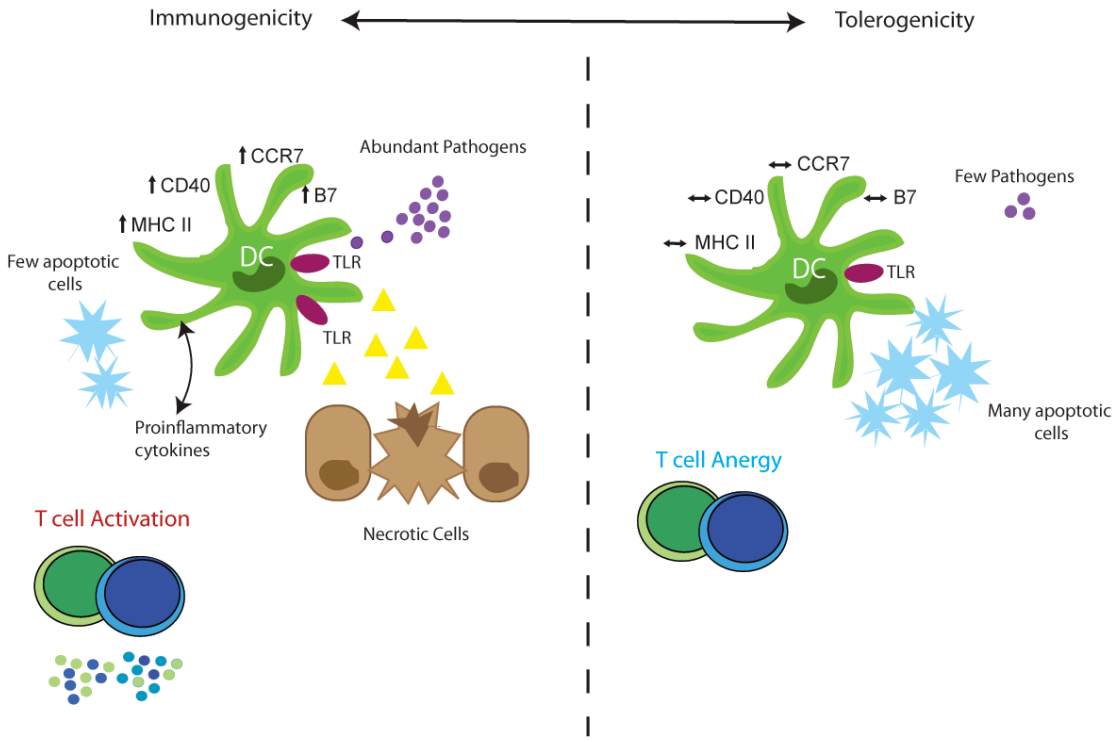


**Figure 1.1. The development of anergy**
As depicted on the left hand panel, immunogenic triggers include the presence of necrotic cells, abundant pathogens signalling to TLRs in the context of inflammatory cytokines and few apoptotic cells. These conditions drive the expression of co-stimulatory markers on DC. If antigen is presented under these conditions it promotes activation of T cells and the initiation of inflammation. In the absence of TLR stimulus and/or in the presence of many apoptotic cells co-stimulatory markers are not upregulated on DC (right hand panel). If T cells recognise antigen presented by DC in this context they do not receive sufficient signal to become activated but are instead anergised. Adapted from[20]

### 1.1.2 Regulatory T cells

In addition to promoting inflammation, T cell subsets have been shown to suppress inflammation and maintain tolerance in the periphery. Treg can suppress cytokine production and proliferation from responder T cells [27-29] as well as suppressing monocytes, macrophages [30], B-cells and dendritic cells [31, 32].

Thymically derived cells capable of suppressing inflammation were first described in experiments by Nishizuka and Sakakura. Mice thymectomised between 2 and 4 days old developed autoimmune disease which was prevented by transferring cells from the thymus or spleen of a non-thymectomised adult mouse [33]. These regulatory T cells were ultimately identified as $CD4^+$ T cells which express the IL-2α receptor, CD25. Transfer of CD25 depleted $CD4^+$ T cells into nude mice resulted in the development of systemic autoimmune pathology. This was prevented if the $CD25^+$ cells were transferred shortly after the $CD25^-$ cells [34].

In humans, $CD25^+$ cells were also found to have suppressive properties [35]. However in contrast to mice, it was demonstrated that only the population of CD4 T cells with the highest expression of CD25 were suppressive in humans [27]. In addition to the expression of high levels of CD25, human Treg have been shown to express low levels of the IL-7 receptor α, CD127. The combination of these two markers is able to discriminate between human regulatory and activated T cells [36]. Furthermore, in contrast to responder T cells, Treg are characterised by low levels of IL-2 and interferon gamma (IFNγ) production [37] and low levels of proliferation when stimulated *in vitro* even in the presence of co-stimulatory signals [28]. In addition to CD25 and CD127, Treg constitutively express CTLA-4 [38], GITR

(glucocorticoid-induced tumour-necrosis factor (TNF)-receptor-related) protein [39] and GARP (glycoprotein A repetitions predominant), an orphan TLR and marker of activated Treg [40]. Membrane bound GARP binds latent TGF-β produced by T cells upon activation and is essential for the surface expression of this cytokine on Treg [40, 41]. As many of the Treg markers discovered to date can at least transiently be expressed by effector T cells, there remains no definitive surface marker of Treg in humans.

#### *1.1.2.1 FOXP3 as a definitive marker of regulatory T cells*

It was the investigation of a unique Treg marker that led to the discovery of Foxp3, part of the forkhead family of transcription factors [42, 43]. Human males with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) and the mouse model of this disease, scurfy, have a mutation in FOXP3. This mutation results in a lack of functioning Treg and the development of autoimmune disease early in life [44]. The importance of Foxp3 for the differentiation, maintenance and suppressive function of Treg was confirmed by retroviral gene transfer of Foxp3 to naïve T cells. Foxp3 transfected cells adopted a regulatory phenotype similar to that of naturally occurring Treg [42].

Foxp3 prevents the conversion of Treg into responder T cells via either the amplification or stabilisation of Treg genes [45], or the suppression of target genes associated with T cell activation [46]. Foxp3 has been shown to interact with a number of other transcription factors including nuclear factor of activated T-cells (NFAT) and runt-related transcription factor 1 (Runx-1). It is thought that Foxp3 binding to NFAT prevents the formation of NFAT-AP1 complexes that are important

for the activation of genes that drive inflammation. Instead Foxp3-NFAT complexes drive Treg-specific genes. Mutation of the NFAT interaction sites in the Foxp3 protein resulted in an inability to induce a Treg gene expression signature and suppressor function [47]. A similar loss of function was seen when Foxp3-Runx-1 interactions were disrupted [48].

FOXP3 gene expression also mediates heritability of Treg phenotype and the generation of induced Treg. Three conserved sequences in the FOXP3 gene, conserved non-coding DNA sequences (CNS) 1, 2 and 3 have been shown to be important for these functions. Knockout of CNS3 results in reduced Foxp3 expression and increased proliferation in Foxp3 precursors, suggesting a role in the thymic development of Treg. CNS2 knockouts showed that this region of the FOXP3 gene is dispensable for Foxp3 expression in thymic Treg but is important in heritability of Foxp3 expression. If $Foxp3^+$ cells from CNS2 knockout animals are made to proliferate, they demonstrate a marked impairment in the maintenance Foxp3. CNS1 is considered entirely dispensable for thymic development but is thought to be important for peripheral induction of Treg. CNS1 knockout results in a failure to accumulate $Foxp3^+$ cells over time, however young mice suffer no pathology, implying that thymically derived Treg are sufficient to prevent autoimmunity [49].

Recently, it has been found that like other Treg markers, FOXP3 can be upregulated by responder T cells after activation but this does not confer a suppressive phenotype [50]. This has cast some doubt on Foxp3 as a definitive marker of Treg. However, recent advances in epigenetic analysis of Treg has found that conventional T cells

that upregulate Foxp3 display only partial demethylation of CpG residues in the proximal promoter of FOXP3, thus identifying methylation status of the Foxp3 promoter as a way of discriminating activated responder T cells from Treg [51].

### 1.1.3 Regulatory T cell development

Treg can be divided into those which develop from immature T cells in the thymus, termed natural Treg (nTreg) or those which are induced from T cells in the periphery in response to various tolerising stimuli (iTreg). Additionally, Treg can be induced *in vitro* and are also referred to as iTreg, though methods of their induction vary (Figure 1.2).

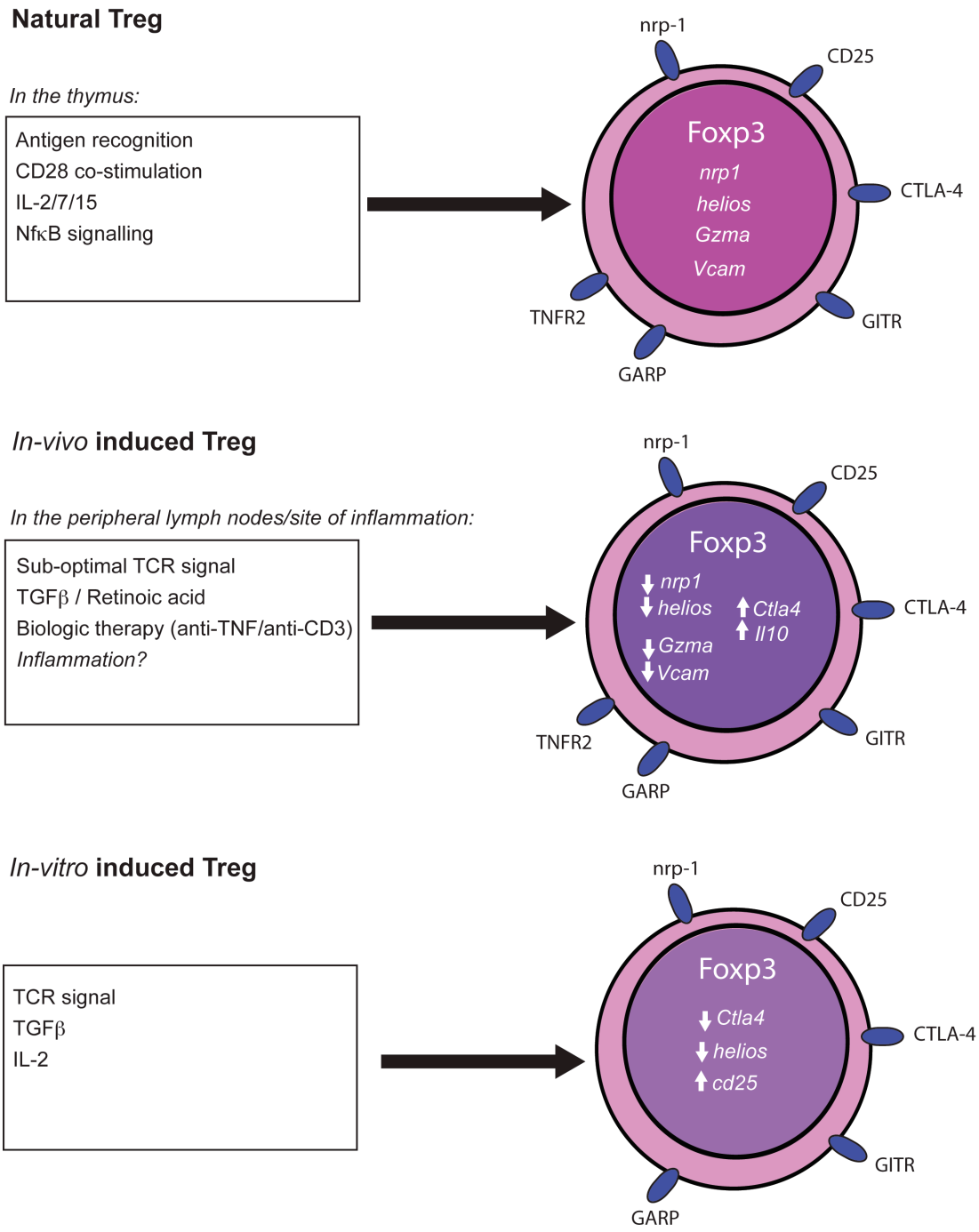


**Figure 1.2. Differences in natural and induced Treg**
Natural Treg (nTreg) develop in the thymus and require a TCR signal, a co-stimulatory signal, gamma chain cytokines and NFκB signalling. On their surface these cells express CD25, GARP, GITR, CTLA-4 and TNFR2. At the genetic level neuropilin-1 (*nrp-1*), Helios, granzyme A (*Gzma*) and vascular cell adhesion protein (*Vcam*) have been shown to be expressed in murine nTreg. *In vivo* induced Treg (iTreg) develop in the lymph node and expand at the site of inflammation. The factors that can drive this induction are listed in the box. Whilst these cells share the same surface markers as nTreg, they express lower levels *Nrp-1*, *Helios*, *Gzma* and *Vcam* but have increased expression of *Ctla-4* and *Il10*. Compared to *in vivo* induced iTreg, *in vitro* iTreg have reduced gene expression of *Helios* and *Ctla-4* but increased expression of *Il2ra*.

### *1.1.3.1 Natural regulatory T cells*

Naturally occurring Treg (nTreg) arise from a pool of moderately self-reactive cells that escape negative selection in the thymus [52, 53]. Indeed, experiments where TCR from Treg were transfected on to responder T cells resulted in a robust expansion of cells and an autoimmune pathology when injected into lymphopaenic recipient mice [54]. Moreover, the crossing of mice expressing an OVA TCR to animals with an AIRE-driven expression of OVA in the thymic epithelium resulted in a substantial increase in nTreg [55]. This suggests that recognising antigen in the thymus is essential for developing a Treg phenotype. Indeed, mice which lack the ability to signal downstream of the TCR fail to generate thymic Treg [19]. Furthermore, there is a decrease in Treg in CD28 deficient mice and mice which are deficient in CD80 or CD86 [56] suggesting that Treg development also requires a co-stimulatory signal.

Additionally, evidence suggests that TCR and CD28 signals induce Treg responsiveness to cytokine signals. Indeed, development of Treg requires the activity of IL-2 and in lesser part IL-7 and IL-15 signalling through common gamma chain cytokine receptors. Mice that are deficient in IL-2 or IL-2 receptor a have a reduction in $Foxp3^+$ thymocytes. Furthermore, mice with a combined deficiency in IL-2, IL-7 and IL-15 or gamma chain cytokine receptors demonstrate a complete ablation of $Foxp3^+$ thymocytes and peripheral $Foxp3^+$ T cells [57, 58]. A suggested candidate for mediating gamma chain cytokine receptor signals to Treg is the signalling protein Signal Transducer and Activator of Transcription (STAT) 5. STAT5 can bind directly to the Foxp3 promoter and CNS2 [57]. Moreover, knockout of STAT5

results in a reduction in Foxp3$^+$ thymocytes [59] whereas constitutive expression of STAT5 results in expansion of Treg cells and a rescue of the CD28 knockout mouse pathology [60].

Downstream of the TCR signal, the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway has been shown to be important for generation of nTreg. Whilst, deletion of the p50 subunit of NFκB resulted in an increase of Foxp3 [61], a loss of function mutation of IKK or deletion of Bcl-10, both activators of NFκB, leads to a reduction in Foxp3$^+$ thymocytes [62]. This suggests that activation of different NFκB family members may have differential effects on Treg fate. Recently it has been shown that enhancing NFκB expression using a constitutively activated IKK transgene led to an increased number of Foxp3$^+$ cells in the thymus. It was found that in Treg from these mice, CNS3 intronic enhancer region of Foxp3 was demethylated. This suggests that NFκB signalling can promote the demethylation associated with stable Foxp3 expression in mature Treg [63]. It is thought that the NFκB family member that contributes to the development of Treg is c-Rel. c-Rel can mediate the formation of a Foxp3 specific enhanceosome, containing c-Rel, NFκB family member p65, NFAT, smad and cAMP response element-binding (CREB) [64]. Moreover, c-Rel-deficient mice have a reduced number of Treg [65].

Thus in order for a Treg to develop in the thymus, an immature T cell must recognise antigen and receive a co-stimulatory signal, but this signal must not be so great that the T cell is deleted. This makes the putative Treg responsive to cytokines, resulting in the activation STAT5 which directly interacts with Foxp3 to drive the Treg

phenotype. Additionally, TCR signals can activate the NFκB family member c-Rel and the consequent formation of a Foxp3 enhanceosome can contribute to the development of Treg.

#### *1.1.3.2 Induced regulatory T cells*

In addition to naturally occurring Treg, under specific conditions expression of Foxp3 and the acquisition of a suppressive phenotype can be induced in peripheral T cells. These cells are termed induced Treg (iTreg) and are typically generated by either a suboptimal signal through the TCR or a strong TCR signal in the presence of large amounts of transforming growth factor beta (TGF-β) [66]. A population of iTreg known as Tr1 cells are an exception in that they do not express Foxp3, but rather like responder T cells, up-regulate Foxp3 after activation. It has been shown that IL-10 in the presence of repeated TCR stimulation can induce Tr1 cells *ex vivo*. Tr1 cells suppress primarily through the production of anti-inflammatory cytokines IL-10 and TGF-β. As a result, once these cells are activated they can suppress in a non-antigen specific manner [67].

Using an animal model that generates tolerance to ingested antigens (oral tolerance), it has been possible to investigate the induction site of iTreg. Oral tolerance is absent in mice without mesenteric lymph nodes [68], suggesting that lymph nodes are vital for iTreg generation. However, the expansion of these iTreg is absent when Treg are unable to traffic to the gut [69]. Thus, Treg need to encounter antigen in the lymph node, but also experience the site of inflammation in order to proliferate and mount

an effective response. Although inflammatory environments differ, one could suggest that this is a general model for the induction of Treg in the periphery.

#### *1.1.3.3 Differences between nTreg and iTreg*

It seems clear that thymic Treg are sufficient to prevent autoimmunity because animals that fail to induce Treg in the periphery do not develop autoimmune pathology [49]. Thus, what is the role of iTreg? Do they work in concert with natural Treg? Or do they have a division of labour? One of the biggest hurdles in answering this question, and indeed in understanding iTreg is that Treg induced in the periphery are indistinguishable from nTreg. However, microarray analysis of murine natural and peripherally induced Treg showed that there are differences at the genetic level between these Treg subsets. Both *Helios* and *Nrp-1* were shown to be expressed at a higher level in nTreg than iTreg [70]. Furthermore, nTreg had a greater expression of *Gzma,* the granzyme A gene, compared to iTreg whilst iTreg were shown to have an increased expression of the *Il10* gene and the *Ctla4* gene. Moreover, a genetic comparison of *in vivo* and *in vitro* iTreg revealed that the *in vitro* induced cells expressed much less *Ctla-4,* the negative regulator of CD28 signalling, than *in vivo* induced Treg but much more *Il2ra* (CD25). Despite this, these two populations of iTreg displayed very similar suppressive ability *in vivo* [71].

Thornton et al. described Helios, an Ikaros transcription factor family member, as a marker for thymic Treg. They showed that all $CD4^+Foxp3^+$ murine thymocytes were $Helios^+$ whereas in the peripheral Treg pool the number of $Helios^+$ Treg was 70% in both humans and mice. Furthermore, it was shown that *in vitro* induced Treg and Treg induced *in vivo* by oral antigen were Helios negative. It seems that under these

conditions Helios could identify thymic Treg [72]. However, there has been some controversy. Like Foxp3 it appears that Helios may be expressed transiently during activation [73], and that this is dependent upon differential stimulation conditions. It was found that induction of Treg from the T cells of RAG-deficient mice, which have no natural Treg, were 50-60% Helios positive if cultured with APC presenting subimmunogenic concentrations of antigen. The authors saw similar results to Thornton et al. if they stimulated cells *in vitro* with anti-CD3 and anti-CD28 [74]. An additional study has shown that the induction of Treg by intra-venous injection of peptide generated cells that express Helios and the continued expression of Helios was associated with continued antigen dependent stimulation [75]. However, while these data may preclude Helios as a definitive marker of thymic Treg, there are still observed differences in Helios gene expression in iTreg [70] so further investigations into the function of Helios in Treg are required to fully elucidate its role.

Neuropilin 1 (Nrp-1) is a surface marker preferentially expressed by Treg [76]. Moreover, ectopic expression of Foxp3 has the capacity to drive Nrp1 expression [76, 77]. Sarris et al. showed that in the absence of inflammation the specific expression of Nrp-1 on Treg increases the contact time with immature DCs. This makes Treg more sensitive to priming with low levels of antigen than responder T cells which have little or no expression of Nrp-1 [78]. Conditional knockout of Nrp-1 does not result in an inflammatory phenotype nor a defect in Treg generation [79]. However, targeted deficiency of Nrp-1 on murine Treg resulted in worse experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Transfecting CD4 T cells from these animals with Nrp-1 was sufficient to suppress proliferation and cytokine production *in vitro* [80].

Many mouse models prove the importance of Treg by transferring naïve T cells into immunodeficient mice, causing disease and then preventing this by the co-transfer of Treg. Using a murine colitis model Haribhai et al. showed that taking naïve cells from Foxp3-deficient mice and co-transferring nTreg from a wildtype mouse does not cure disease. In these experiments disease could only be cured when nTreg were transferred together with *in vitro* induced Treg [70]. Moreover, similar experiments showed that lymphoproliferative disease is only completely absent in Foxp3-deficient mice adoptively transferred with wildtype T cells upon the conversion of a proportion of responder T cells to iTreg [71]. This suggests that nTreg and iTreg have specific distinct roles in suppressing inflammation.

### 1.1.4 Mechanisms of regulatory T cell suppression

There are a number of proposed mechanisms of Treg suppression (Figure 1.3). These include both contact-dependent and soluble factor-dependent pathways.

#### *1.1.4.1 IL-2 deprivation*

It has been suggested that high expression of the IL-2 receptor α (CD25) on Treg could outcompete T cells for IL-2, resulting in apoptosis of pathogenic responder cells. Indeed, pre-treatment of responder T cells with IL-2 reduced levels of apoptosis when cells were subsequently cultured with Treg [81].

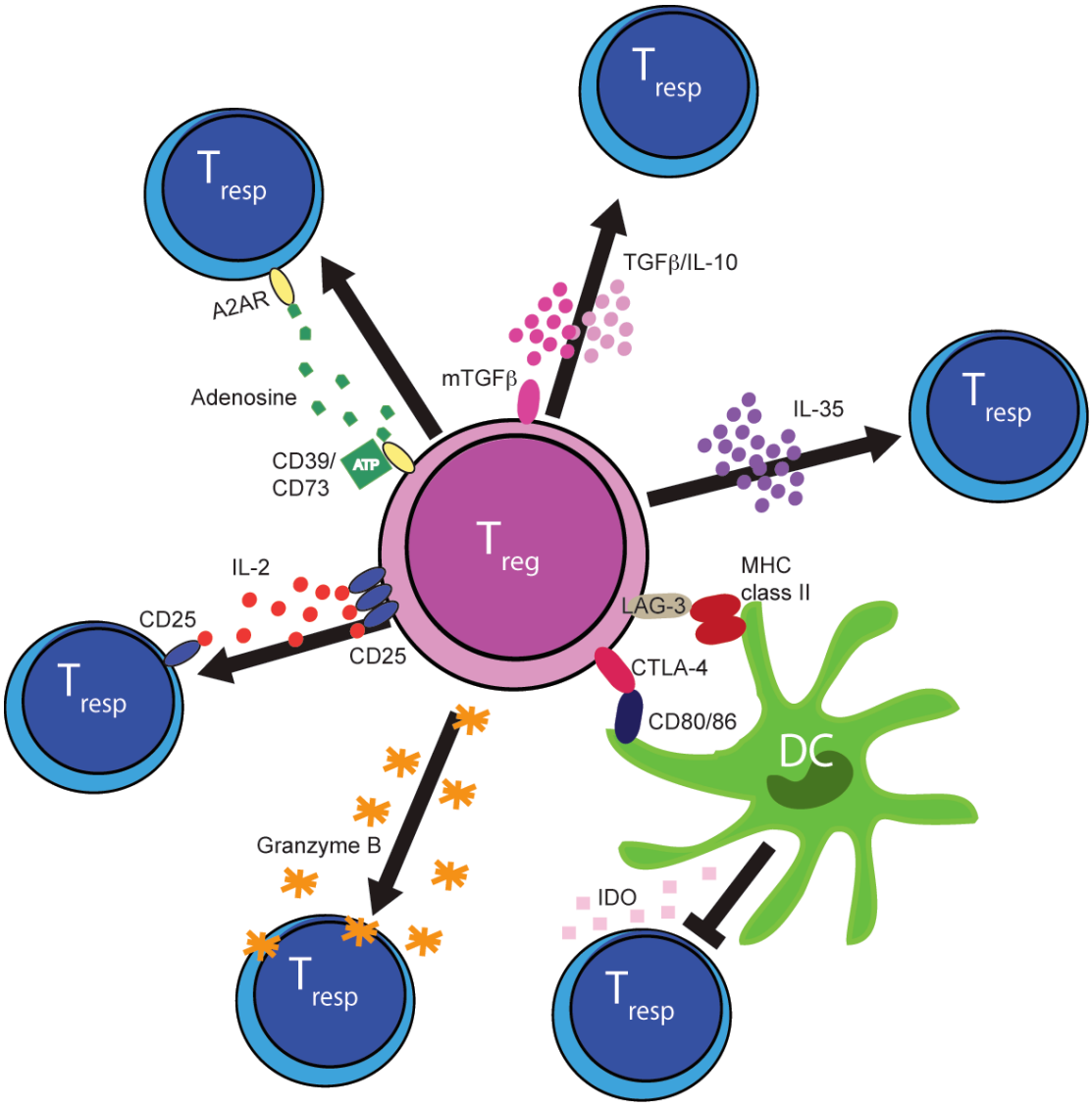


**Figure 1.3. Depiction of different putative mechanisms of regulatory T cell suppression.**
The suppressive mechanisms utilised by regulatory T cells (Treg) to suppress responder T cells (Tresp) include the production of inhibitory cytokines such as IL-10, IL-35 and the soluble and membrane bound isoforms of transforming growth factor beta (TGFβ and mTGFβrespectively), (2) cell killing e.g. granzyme B or IL-2 deprivation through elevated expression of IL-2Rα (CD25) on Treg, (3) enzyme activity of CD39 and CD73 catalysing the breakdown of adenosine triphosphate (ATP) to adenosine which can signal to Tresp via their expression of adenosine A2A receptor (A2AR), (4) inhibition through effects on dendritic cells (DC), including the triggering of indoleamine 2,3-dioxygenase (IDO) production, (5) contact-dependent inhibition via cytotoxic T-lymphocyte antigen 4 (CTLA-4) or lymphocyte-activation gene 3 (LAG-3).

#### *1.1.4.2 The production of anti-inflammatory cytokines interleukin-10, transforming growth factor beta and interleukin-35*

Cytokines produced by Treg have also been shown to be important for suppression. Treg express TGF-β-1 on their surface and this can be increased by stimulation with anti-CD3 *in vitro* [82]. TGF-β in either the membrane bound form [82] or soluble TGF-β produced by Treg was shown to be required to inhibit Th1 cell differentiation and inflammatory bowel disease [83]. Moreover, TGF-β knockout mice have multifocal inflammatory disease [84]. Similarly, ablation of IL-10 in $Foxp3^+$ cells showed that IL-10 was important for maintaining tolerance at mucosal surfaces [85]. Furthermore, IL-10 and TGF-β have been shown to mediate the suppressive function of Treg induced by anti- tumour necrosis factor (TNF) therapy [86].

More recently Treg producing IL-35 have been described. IL-35 is part of the IL-12 family of cytokines, made up of a p35 subunit and EBI3 (Epstein-Barr virus induced gene 3). In the absence of either subunit, murine Treg which have been adoptively transferred into lymphopaenic recipients fail to control proliferation and the animals develop inflammatory bowel disease [87]. Moreover, the addition of recombinant IL-35 can inhibit both human and murine T cell proliferation. In addition to suppressing T cells, IL-35 is sufficient to convert responder T cells into hyporesponsive, suppressive but Foxp3 negative Treg (iTr35). These cells suppress via the production of IL-35 but not IL-10 or TGF-β. Despite this, IL-10 appears to be required for the optimal conversion of responder T cells to iTr35 cells. IL-10 mutant mice or blocking antibodies result in an impairment in conversion but not suppressive function of iTr35 [88]. Using transwell experiments where murine Treg and responder T cells were cultured together in the upper chamber and responder T cells

were cultured in the lower chamber separated by a permeable membrane; it was shown that optimal IL-35 production was only induced from Treg that had made direct contact with responder T cells. This suggests that Treg can simultaneously utilise both contact dependent and soluble factor dependent pathways. Interestingly, in this assay the neutralisation of IL-10 reduced the suppressive ability of nTreg. This suggests that IL-35 and IL-10 can function in synergy to suppress responder T cells after contact dependent interaction [89].

It has been suggested that IL-35-induced Treg can mediate the suppression of IL-17 production, which is largely outside the control of nTreg [90]. Indeed, IL-35 has been used therapeutically to treat a mouse model of RA, collagen induced arthritis (CIA), and works through the expansion of Treg and the suppression of IL-17 [91]. There is some debate however about the role of IL-35 in human Treg. Some studies have shown that human nTreg do not make IL-35 [92] whilst others have shown IL-35 production by Treg mediates contact independent suppression and conversion of responder T cells to iTr35 cells [93].

#### *1.1.4.3 CD39*

An excess of adenosine triphosphate (ATP) is released by cells as they die and drives inflammation through the recruitment and maturation of dendritic cells which express ATP receptors [94]. In turn, the breakdown of ATP into adenosine by the endonucleotidases CD39 and CD73 can inhibit pro-inflammatory responses [95]. In mice CD39 and CD73 are co-expressed on Treg and CD39 null mice fail to prevent allograft rejection *in vivo* [96]. Moreover, CD39 knockout animals show increased susceptibility to colitis and this has been linked to a CD39 SNP associated with

susceptibility to colitis in humans [97]. Similarly, CD73 knockout animals have increased expression of inflammatory cytokines in a transplantation model. This deficiency in CD73 is rescued by the addition of an agonist of the adenosine A2A receptor [98]. Moreover, a study has shown that Treg cannot suppress responder T cells which lack A2A receptor expression [99]. Thus, the capacity of CD39 and CD73 to mediate the suppression of inflammatory responses requires adenosine receptor expression on the target cell.

In humans, however, it is unclear if CD39 and CD73 are co-expressed on Treg. One study found that CD39 was expressed on Treg, but CD73 was absent [100], whilst another found that only 0.2% of whole T cells co-expressed CD73 and CD39 [101]. By contrast, others have detected co-expression of CD39 and CD73 on human Treg [102, 103]. Despite this controversy, CD39 activity has been implicated in the suppressive function of human Treg. Gandhi et al. showed that iTreg suppression of responder T cells was reversed by a CD39 specific blocking antibody [104]. Furthermore, Fletcher et al. described a subset of natural Treg that expressed CD39. Upon isolation these cells could suppress IL-17, a characteristic not observed in whole Treg. Suppression was TGF-β-independent and could be imitated by the addition of adenosine. However, inhibitors of adenosine breakdown or binding did not reverse the suppressive ability of $CD39^+$ Treg [105]. Thus CD39 expression identified a population of IL-17 suppressing cells and adenosine demonstrated the capacity to regulate IL-17 production. However, it is still unclear if the generation of adenosine by CD39 activity on Treg was required for the suppression of IL-17 responses in this study.

#### *1.1.4.4 Cytotoxic T-Lymphocyte Antigen 4*

CTLA-4, like CD28 can bind CD80 and CD86. It has been shown that in CD28 deficient mice, cells which express CTLA-4 cannot provide a co-stimulatory signal to the T cell [106]. Moreover, mice that are deficient in CTLA-4 suffer fatal autoimmune pathology [107, 108] implicating CTLA-4 as a negative regulator of T cell activation. In contrast to CD28, which is constitutively expressed on T cells, CTLA-4 is only constitutively expressed on Treg [109, 110]. The interaction of CTLA-4 with CD80 and CD86 on DCs has been shown to down modulate their expression [111]. Indeed, autoimmunity in mice with a selective loss of CTLA-4 on Treg is prevented by the blockade of CD80/86 by CTLA-4Ig [112]. Quite recently it has been shown that CTLA-4 functions by efficiently binding to B7 family members and trans-endocytosing the whole molecule. This rips the co-stimulatory molecules from the surface of APCs so that they fail to effectively stimulate T cells [113].

Whilst this is perhaps the major function of CTLA-4, it has been suggested that this molecule can contribute to the induction of tolerance through other mechanisms. There is evidence that signalling through CD80/86 induces indoleamine 2,3-dioxygenase (IDO) which can degrade tryptophan to by-products which suppress T cell proliferation [114]. Indeed pharmacological inhibition of IDO abrogates the protective effects of CTLA-4 Ig [115]. However IDO deficient mice do not resemble CTLA-4 deficient mice and lack a dysregulated immune phenotype [116]. So tryptophan breakdown may only comprise a small facet of CTLA-4 biology.

#### *1.1.4.5 Lymphocyte-activation gene 3 and Granzyme*

Another surface marker thought to mediate Treg suppression is lymphocyte-activation gene 3 (LAG-3), a CD4 homologue that can bind MHC class II. LAG-3-CD4 interaction can prevent DC maturation and co-stimulatory molecule expression. Natural Treg express LAG-3 upon activation and this is enhanced in the presence of responder T cells. Moreover, LAG-3-deficient mice have impaired regulatory activity. Ectopic expression of LAG-3 on CD4 T cells reduces their proliferation and confers them with suppressive ability. Despite having greater affinity for MHC than CD4, LAG-3 does not appear to outcompete CD4 for MHC. In fact the mechanism of LAG-3 mediated suppression is unknown, though there is a correlation between LAG-3 expression and IL-10 mRNA in iTreg [117].

Treg have also been show to suppress by granzyme B-mediated cytolysis of effector cells or APCs [31, 118]. *In vivo* it was shown that granzyme B was required for maintenance of skin allograft transplant and the suppression of tumour clearance [118, 119].

Thus, it is unclear exactly which mechanisms Treg use *in vivo*. It is possible that they utilise all of the mechanisms described above and the method of suppression is dependent upon environmental cues.

### 1.1.5 Regulatory T cells in rheumatoid arthritis

As previously discussed, Treg are vital to the prevention of autoimmunity. Thus, it was hypothesised that Treg from patients with active RA would demonstrate a defect in number or suppressive function. Some studies have described a decrease in circulating Treg in patients with RA compared to healthy controls [120-122], suggesting a numerical defect in Treg may contribute to disease pathogenesis. However, other groups have shown no difference [123-126] and some have even shown an increase in Treg in RA patients compared to healthy controls [127, 128]. An increase in $FOXP3^+$ cells has also been reported in the synovial fluid of patients with RA, and these cells were shown to be potent suppressors *in vitro*. However, the activated T cells from this site appear to be resistant to suppression [127], which explains the inability of Treg to dampen inflammation in the joint.

Upon investigation of the suppressive function of Treg from patients with RA, Ehrenstein et al. showed that whilst Treg from patients with active RA were proficient suppressors of proliferation, in contrast to healthy controls, Treg from these patients were unable to suppress the production of TNF and IFNγ from responder T cells *in vitro*. These data have since been confirmed in studies by two independent groups [129, 130]. Thus, whilst there is some controversy regarding the numbers of circulating Treg in patients with active RA, there is a consensus that Treg from these patients are defective. This defect has been associated with reduced expression of CTLA-4 on Treg from patients with active RA compared to Treg from healthy controls. Indeed, forced expression of CTLA-4 by PMA treatment was sufficient to restore suppressive capacity to Treg from patients with active RA [37].

### 1.1.6 Factors which contribute to the breach of tolerance in rheumatoid arthritis

#### *1.1.6.1 Genetic polymorphisms associated with rheumatoid arthritis*

Many of the SNPs associated with the development of RA (Table 1.2, pg 52) code for proteins that, if altered, could change the very nature of the immune response. Thus providing rare insight into the pathways that lead to the generation of autoimmunity.

Interestingly, a number of these SNPs can be grouped according to their role in the immune response. Human leukocyte antigen (HLA)-DR and protein tyrosine phosphatase, non-receptor type 22 (PTPN22) are important for T cell activation, TNF receptor-associated factor 1 (TRAF-1), A20 (tumour necrosis factor, alpha-induced protein 3) and CD40 are members of the TNF receptor signalling family and IRF5 and STAT4 are important for cytokine responses. Indeed T cell hyperactivation, TNF activity and widespread cytokine production are key features of RA pathogenesis. Moreover, antigen presentation (HLA-DR), T cell signalling (PTPN22) and T cell trafficking (CCL21) are important features of central and peripheral tolerance. Thus alterations of these pathways caused by RA-associated SNPs could undermine tolerogenic mechanisms and prolong inflammation.

| SNP | Encodes | Association of molecule with disease |
|---|---|---|
| HLA-DR | MHC class II molecules which present antigen to CD4 T cells. | All HLA-DR molecules associated with RA share an amino acid residue on one side of the antigen-binding site, the shared epitope. |
| PTPN22 | Protein tyrosine phosphatase that deactivates Lck an important mediator of T cell signalling. | T cells with the RA-associated SNP display potent negative regulation of T cell signalling. Associated with rheumatoid factor positive RA and an earlier disease onset. |
| TRAF-1 | Negative regulator of TNF receptor signalling. | Animals deficient in TRAF-1 have increased proliferation dependent on TNFR2 and increased cell death dependent on TNFR1. |
| A20 | Negative regulator of NFκB activation in response to TNF or TCR signalling. | Conditional myeloid cell knockout of A20 leads to the development of a RA-like disease. A20 is repressed in RA SNPs. |
| CD40 | Surface molecule required for T cell dependent antibody response, induction of co-stimulatory molecules on APC and modulating apoptosis. | CD40 SNP in patients with RA is associated with a higher rate of joint destruction in ACPA-positive patients [131]. |
| STAT4 | Signalling molecule expressed in Th1 cells in response to IL-12. | RA associated SNP is located in the third intron of STAT4. Individuals homozygous for this SNP are 60% more likely to develop RA |
| IRF5 | Regulates type I interferons in response to stimulation via TLR7 and TLR9. | SNP is in the promoter region of the gene and is associated with ACPA negative patients. There are increased type I interferons in RA patients. |
| PADI4 | Encodes PAD enzymes responsible for the conversion of arginine to citrulline under oxidative stress, differentiation and apoptosis. | Weak genetic association with RA, despite ACPA antibodies being a key feature of RA pathogenesis. |
| CTLA4 | Negatively modulates CD4 co-stimulation through trans-endocytosis of CD80/CD86 on antigen presenting cells. | Weak genetic association with RA in Caucasians, but RA patients have reduced CTLA-4 expression on Treg and CTLA-4 Ig is an effective therapy in RA. |
| C5 | Member of the complement pathway. C5a is a potent inflammatory mediator and C5b is important for the formation of membrane attack complexes. | C5aR expression on RA synovicytes showed correlation with the number of swollen joints. An anti-C5 inhibitor is presently in trials for the treatment of RA. |
| CCL21 | The ligand of CCR7, important for the homing of T cells and DC to secondary lymphoid organs. | Synovial CCL21 induces vascularisation and is modulated by TNF. Implicated in the formation of lymphoid tissue. |

**Table 1.2 Single Nucleotide Polymorphisms associated with the development of RA**

SNPs can be divided into those that are implicated in antigen presentation and T cell receptor signalling (yellow), TNF receptor signalling pathways (blue), cellular differentiation factors (pink), those with weak genetic association but with a role in RA pathogenesis (purple) and inflammatory amplifiers (green).

### *1.1.6.2 The role of infection in the development of rheumatoid arthritis*

There has long been considered a link between infection and the development of autoimmunity. Mice that spontaneously develop EAE do not do so in pathogen free conditions [132], thus highlighting that genetic susceptibility is not sufficient for the development of autoimmunity. Microbial infections can initiate or enhance susceptibility to autoimmunity by stimulating a strong inflammatory response. Moreover, microbial fragments can directly stimulate fibroblast-like synovial cells (FLS) via their expression of toll-like receptors (TLRs). Stimulation of FLS with a TLR2 agonist stimulated the production of chemokines such as CXCL8, CCL5 and CCL8 for the recruitment of inflammatory cells [133]. In RA, parovirus B19 is thought to be an infection associated with the development of disease. Parovirus B19 genetic material was detected in the synovial membrane of 75% of RA patients [134] compared to just 17% in patients with osteoarthritis. Similarly, Epstein barr virus material is found in 34% of RA patients and just 10% of healthy controls [135].

Microbes can also share epitopes of self-antigens in a phenomenon called molecular mimicry. These epitopes initiate clonal expansion of T cells in response to the microbial infection, but inflammation is prolonged because the antigen is also expressed on the tissues of the infected individual. *Porphyromonas gingivalis* (*P.gingivalis*) is associated with the development of RA. This oral bacterium can citrullinate the bacterial form of enolase [136]. This may in turn drive a cross-reaction with human citrullinated enolases. Citrullinated proteins are thought to be important for the initiation of RA (discussed in section 1.1.71). Indeed, mice with an RA susceptible MHC (DR4-IE transgenic mice) immunised with *P. gingivalis*-

derived enolase developed an autoimmune response upon exposure to mammalian α-enolase [137]. Thus, infection could initiate an aberrant immune response to self-tissue.

#### *1.1.6.3 Environmental, hormonal and stochastic factors associated with the development of rheumatoid arthritis*

There are many environmental factors associated with the development of RA including silica dust and cigarette smoke. These factors are usually associated with tissue stress and damage. Indeed, expression of TLR3 on FLS allows these cells to respond directly to RNA from necrotic cells linking tissue trauma to the development of proliferative, invasive synovial cells in RA [138].

Smoking has been widely described as a risk factor for RA [139] and there is evidence of increased citrullinated proteins in the bronchoalveolar lavage of smokers with pulmonary infections [140]. It has been found that cyanide in cigarette smoke can activate thiocyanate ions in lung tissue, which in turn can be broken down into homocitrulline. Moreover, increased levels of homocitrulline peptides have been associated with arthritides in which patients test positive for anti-citrullinated protein antibodies (ACPA) [141]. Interestingly, smoking is only a risk factor for RA in individuals who carry HLA-DRB1 shared epitope alleles [142]. Indeed, citrulline has been shown to fit directly into this conserved region which has a strong association with the development of RA [143]. Moreover, MHC class II susceptibility genes for RA have been found to correlate with production of ACPA

[144]. Thus, this provides a direct pathway for citrullinated proteins to trigger RA in genetically susceptible individuals.

There is expression of sex hormone receptors on monocytes and *in vitro* physiological levels of oestrogen stimulate IL-1 production, but high levels reduce IL-1 production by RA monocytes [145]. Moreover, IL-6, TNF and IL-1 can drive the activation of the stress system and the production of glucocorticoids from the adrenal gland which can in turn modulate the immune response [146].

### 1.1.7 Proposed rheumatoid arthritis antigens

The final step in the initiation of RA is the presentation of autologous antigen to T cells. However, the antigen that triggers disease is unknown. Moreover, it is unlikely to be the same for all patients with RA. It is clear that the immune response commences some time before the onset of clinical symptoms because a study of blood donors who later went on to develop RA found that rheumatoid factor antibodies and ACPA were present up to 3 years before diagnosis [12, 13]. This also implicates citrullinated proteins or immune complexes as possible triggers of RA and is discussed more below. In addition, there are a number of endogenous antigens that are possible candidates for driving RA. Proteins such as type II collagen [147], proteoglycans [148], aggrecan [149] and heat shock proteins [150] have been shown to drive responses in lymphocytes from patients with RA more readily than healthy controls.

#### *1.1.7.1 Citrullinated proteins*

Eighty percent of RA patients present with anti-citrullinated protein antibodies (ACPA) [151] and serum levels may predict disease activity [152]. Citrullination is a post-translational conversion of an arginine residue to citrulline. As a result, citrullinated proteins are unlikely to have been presented to developing T cells in the thymus, which increases the chances of citrullinated proteins initiating a breach of tolerance. The conversion of arginine to citrulline is important for gene regulation, nuclear lamina deorganisation, nucleosomal collapse, vimentin filament collapse, organising the matrix of keratins, protein demethylation and vimentin regulation [153]. PAD4 is a peptidylarginine deiminase, part of a family of enzymes responsible for the conversion of arginine to citrulline. A polymorphism in PADI4, the gene that codes PAD4, has a weak association with the development of RA. Furthermore, it has recently been shown that a general PAD inhibitor was able to reduce the severity of CIA, reducing citrullination, anti-mouse type II collagen antibodies, histopathology scores and total complement C3 deposition [154].

Citrullinated proteins are detectable in the synovium of RA patients and include citrullinated fibrin, vimentin, type II collagenase and alpha-enolase [155-158]. Indeed, in the RA synovium, conditions of oxidative stress and cell death are common [14, 159] and can drive production of intracellular calcium. High levels of intracellular calcium can trigger the activation of PAD enzymes which catalyse the citrullination of proteins including vimentin. Loss of membrane integrity associated with cellular necrosis could expose this protein to uptake by APCs. In individuals with the RA susceptible HLA allele the citrulline residue may be recognised by the

shared epitope region and trigger an autoimmune response [153]. In DR4-IE transgenic mice, only citrullinated fibrinogen could cause arthritis; unmodified fibrinogen did not drive disease. Moreover, it was shown that intra-articular injection of antigen to the joint drove disease and this was absent in animals that were injected elsewhere. Thus, citrullinated proteins specifically drive an arthritis-like disease, but these proteins must be expressed in the joint [160]. This implies that even if environmental factors such as smoking generate citrullinated proteins in the lung tissue, the immune response must be targeted to the joint by an additional factor.

Thus, conditions in the synovium such as stress, apoptosis and cellular differentiation could activate PADS, increasing citrullination and the chances of presentation of citrullinated proteins to T cells.

#### *1.1.7.2 Rheumatoid Factor*

Rheumatoid factor (RF) autoantibodies are found in approximately 80% of RA patients [161] and react with the Fc portion of IgG [162]. Rheumatoid factor production is induced by polyclonal activation of B cells and exposure to antigen-antibody complexes. IgM rheumatoid factor can bind to the relatively unspecific IgG induced by polyclonal activation increasing avidity for pathogen [163]. Evidence suggests that RF is not specific for RA but rather is indicative of an ongoing immune response. This is based upon the fact that 4-30% of healthy individuals can express RF [164] and RF is found to be increased in other autoimmune and rheumatic diseases [163]. Despite this, there is little doubt that RF contributes to RA pathology. IgM RF is highly multivalent and thus forms large immune complexes that are

poorly soluble and become trapped in the synovium. Not only does this activate the complement system but it stimulates phagocytosis and the activation of neutrophils which produce hydrolytic enzymes contributing to tissue destruction [165]. Epstein Barr, an infection associated with the development of RA, can induce rheumatoid factor synthesis from both healthy cells and cells from patients with RA [166]. Thus, RF may be induced by infection, which in the presence of RA susceptibility genes and citrullinated peptide-antibody complexes that are present early in disease, may link infection to the development of RA.

## 1.2 Inflammation

### 1.2.1 T cells:

Once it was established that there were two types of circulating lymphocytes, the thymus derived T cells and the bone marrow derived B cells, it soon became clear that these two populations influenced one another. In order for B cells to produce antibodies against so-called 'thymus dependent antigens' they required the presence of T cells; these cells were named helper T cells. In 1974, Hunter and Kettman showed that the supernatant from a culture of activated T cells was able to stimulate B cell proliferation and differentiation [167]. This was one of the earliest descriptions of the activity of cytokines, now known to be pleiotropic soluble factors vital for cell-to-cell signalling.

#### *1.2.1.1 The Th1/Th2 paradigm*

In 1986 Mosmann et al. first described two distinct subsets of T helper cells Th1 and Th2, defined by their cytokine profile. Each subset was found to offer help to B cells, but the nature of the help differed [168]. Thus, it became clear that different Th subsets are necessary for mounting appropriate responses to different pathogens. Th2 cells are induced by IL-4, but also produce IL-4, IL-5 and IL-13. They are characterised by the high expression of the transcription factor GATA-3 and are important for immunity to extracellular pathogens. An aberrant Th2 response is associated with the development of allergy by assisting B cells in the production of IgE. Th1 cells are important for cell-mediated immunity; they produce IFNγ and are induced from naïve T cells by IL-12. T-bet, part of the T-box family of transcription

factors, is known as the master transcription factor of Th1 cells. It is important for the production of IFNγ and the stabilisation of the Th1 phenotype [169]. Furthermore, the ectopic expression of T-bet into polarised Th2 cells results in the production of IFNγ and suppression of IL-4 and IL-5 [170] (Figure 1.4).

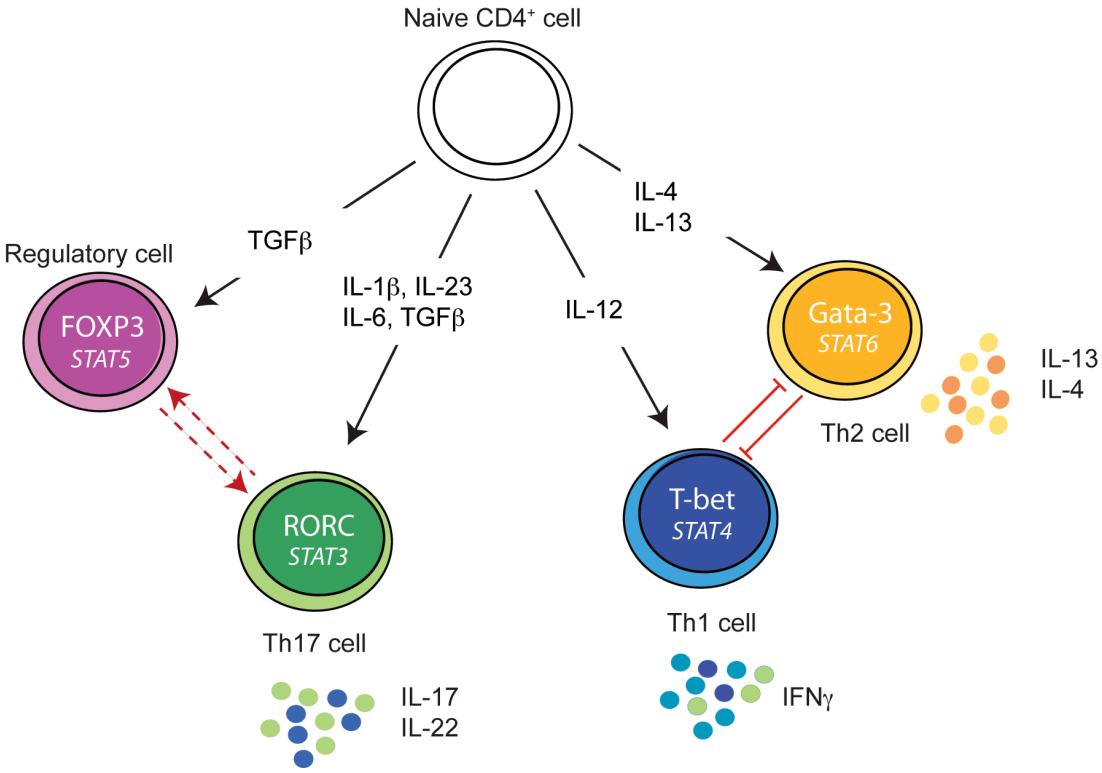


**Figure 1.4. T helper cell subsets**
In the presence of IL-4 and IL-13 naive T cells up-regulate GATA-3 and STAT6 and begin producing IL-13 and IL-4. In the presence of IL-12 naive T cells up-regulate T-bet and STAT4 and produce INFγ. These two cell fates are mutually exclusive. Alternatively, if naive T cells are activated in the presence of TGF-β with some combination of IL-1β, IL-23 and IL-6 cells up regulate RORC (in humans) and STAT3 and begin producing IL-17 and IL-22. In contrast, TGF-β alone is sufficient to induce regulatory cells that express FOXP3 and STAT5.

STAT proteins are vital in T cell lineage commitment and different members of the STAT family have been shown to be associated with different T helper cell subsets. STAT6 binds the IL-4Rα gene [171], which allows up-regulation of this receptor, increased responsiveness to IL-4 and a skewing towards a Th2 phenotype. STAT6 knockout mice demonstrate impaired proliferation in response to IL-4 and a complete abrogation of IL-4 receptor expression. Furthermore, T cells from these mice fail to differentiate into Th2 cells in the presence of IL-4 or IL-13 [172, 173]. STAT4 is associated with a Th1 phenotype. It has been shown that STAT4-deficient mice demonstrate disruption of IL-12-mediated T cell functions including IFNγ production and differentiation of Th1 cells. Moreover, T cells from these mice are skewed towards a Th2 phenotype [174, 175].

#### *1.2.1.2 The role of T cells in the pathogenesis of rheumatoid arthritis*

In addition to helping B cells produce antibody, inflammatory cytokines produced by T cells are directly implicated in the pathogenesis of RA. Studies in mice with CIA suggested that arthritis in these animals was mediated primarily by Th1 cells. Levels of IFNγ in the joints of diseased animals were directly correlated with disease severity. This was matched by an almost complete suppression of Th2 cell cytokines [176]. Subsequent studies showed that CIA mice treated with IL-12 developed exacerbated disease and that neutralisation of this cytokine reduced disease severity [177]. Whilst models in which the p40 subunit of IL-12 was knocked out mirrored the effects of neutralising IL-12, disease models in which the p35 subunit of IL-12 was knocked out resulted in exacerbation of disease [178]. This contradiction was explained by the discovery of an interleukin made up of the p40 subunit of IL-12

with a novel p19 subunit, named IL-23 [179]. This suggested that IL-23 could be as fundamental in autoimmune pathology as IL-12. In fact, subsequent experiments in which the p19 subunit of IL-23 was ablated showed that it was IL-23 and not IL-12 which mediated autoimmunity in a mouse model of colitis [180]. Like IL-12, IL-23 was found to activate STAT4 but to a lesser extent [179, 181]. Rather, STAT3 was found to transduce intracellular signals in response to IL-23 [181].

STAT3 is the only STAT where knockout is embryonically lethal indicating an important role for STAT3 in early development [182]. Immunoprecipitation studies showed that STAT3 phosphorylation is induced by IL-6 and epidermal growth factor but not IFNγ [183]. Interestingly both IL-6 and IL-23 have been shown to be important for the induction and maintenance of a relatively newly described T helper cell subset, Th17. Moreover, it was found that retroviral expression of STAT3 enhanced the induction of Th17 cells and STAT3 deficiency reduced expression of retinoic-acid-receptor-related orphan receptors (ROR)γt [184], the Th17 cell-associated transcription factor [185]. Thus STAT3 is considered to be vital for the differentiation of Th17 cells.

### 1.2.2 Th17 cells

T cells producing IL-17A were first described in 1995 [186] but it was 10 years later before these cells were defined as a distinct T helper cell subset. Experiments by Harrington and colleagues found that the induction of $CD4^+$ T cells that could produce IL-17 was potently inhibited by IFNγ and IL-4. In addition they showed that in the absence of Th1 or Th2 cytokines, naïve T cells became Th17 cells and this was

independent of T-bet, STAT1, STAT4 or STAT6 [187]. RORγt was shown to be necessary for the Th17 cell phenotype because mice reconstituted with the bone marrow of RORγt-deficient mice had reduced Th17 cells and transduction of cells from these animals with RORγt reversed this defect [185].

In mice, Th17 cells can be induced from naïve $CD4^+$ T cells cultured in DC-conditioned media. This induction is prevented by blocking TGF-β and IL-6. The importance of these cytokines for Th17 cell differentiation was confirmed by adding IL-6 and TGF-β to stimulated naïve CD4 cells, resulting in their differentiation into Th17 cells. The addition of IL-1β further augmented this induction [188, 189]. In humans, the exact combination of cytokines required to induce Th17 cells *in vitro* is still somewhat controversial. It is clear that TGF-β is essential for the induction of human Th17 cells [190-192]. TGF-β is sufficient to induce RORγt expression but inhibits its function and this inhibition is only reversed in the presence of inflammatory cytokines [190]. Thus, it has been shown that in addition to TGF-β, the differentiation of human Th17 cells can be achieved with inflammatory cytokines including IL-1β, IL-23, IL-21, IL-6 or combinations thereof [190-193]. This has been disputed however, as one study has shown that neither IL-6, IL-21 nor TGF-β was sufficient for the induction of human Th17 cells but rather, T cells required contact with activated monocytes in the context of TCR ligation [194].

Th17 cells mediate the immune response to fungal infections and in addition to IL-17A have been shown to produce IL-17F, IL-21, IL-22, IL-26, TNFα and CCL20 [193, 195, 196]. Th17 cells have since been identified as key mediators of inflammation in autoimmune disease [197]. IL-17 levels have been found to be

elevated in the periphery of patients with RA [198] and IL-17 has been shown to contribute to joint degradation in these patients [199]. Moreover, activated monocytes from the joints of patients with RA have been shown to promote Th17 responses [200]. Indeed the ability of monocytes to contribute to the induction of Th17 cells during inflammation has been reinforced by a study showing that in RA levels of TNF producing $CD14^{hi}CD16^{+}$ monocyte numbers correlate with Th17 cell numbers [201]. Thus, monocyte-driven Th17 cell development could play an important role in RA pathogenesis.

### 1.2.3 Th17 cell stability

Studies have identified cells producing both IL-17 and cytokines associated with other T helper cell subsets. These Th17 "dual producers" are seen most frequently in disease settings, such as autoimmunity [202] or allergy [203]. Indeed, the adoptive transfer of Th17 cells to lymphopaenic mice drives the conversion of Th17 cells to IFNγ producers and triggers an IFNγ-mediated diabetes [204]. Furthermore, fate mapping IL-17-producing cells showed that Th17 cells are induced during EAE but that over time these cells stop producing IL-17 and make other cytokines including IFNγ. Indeed, it was shown that the primary producers of IFNγ in this model had previously produced IL-17 and that these 'ex-Th17' cells retained Th17 cell genetic markers [205]. This ability for inflammation to convert Th17 cells may explain why Treg do not demonstrate the capacity to suppress Th17 cells [206]. Thus conditions in the synovium may favour the differentiation of Th17 cells, which are outside the scope of natural Treg. In the absence of negative regulation by conversion to other

cell types Th17 cells could drive inflammation and joint damage almost entirely unchecked.

### 1.2.4 The relationship between regulatory T cells and Th17 cells

In 2006 Bertelli et al. were the first group to suggest that there was a reciprocal relationship between the development of Treg and Th17 cells [189]. In the presence of inflammatory cytokines, TGF-β can induce Th17 cells and in the absence of an inflammatory cytokine milieu the induction of Treg is favoured [188]. More recently it has been shown that Foxp3 can directly interact with the master regulator of Th17 cells, RORγt, in order to suppress the transcription of IL-17 [207]. IL-17 levels are higher in the periphery of patients with active RA than healthy controls [208]. Thus the link between these two functionally distinct cell subsets may have some influence in the pathogenesis of RA.

The aryl hydrocarbon receptor (AhR), a member of a family of environment-sensing transcription factors, has been shown to be important for both Th17 and Treg differentiation [209]. The influence of AhR suggests that both of these cell subsets may be heavily modulated by an inflammatory environment. Naïve T cells from *ahr*-null mice did not form Treg *in vitro*, whilst Th17 cells were reduced in *ahr*-null mice upon the induction of EAE [210, 211]. In a murine colitis model, treatment of mice with a high affinity AhR ligand, TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), ameliorated disease. This correlated with an increase in Treg and a decrease in Th17 cells through demethylation of CpG regions in the FOXP3 promoter and an increased methylation at the IL-17A promoter [212]. Moreover, TCDD has been found to

ameliorate disease in NOD mice through the induction of Treg [213]. In human cells, AhR activation promoted the differentiation of Foxp3 negative Tr1 cells, which suppressed responder T cells via IL-10 and granzyme B. Furthermore, if the same starting population of cells was treated with an AhR ligand in the presence of TGF-β, it induced a subset of Treg which suppressed via the activity of CD39 [104].

In addition to responding to environmental cues via AhR, it appears that a metabolic sensor, hypoxia inducible factor 1 (HIF-1), can modulate Treg and Th17 cell balance. Mice with HIF-1α deficient T cells are resistant to the induction of EAE and this is associated with a reduction in Th17 cells and an increase in Treg [214, 215]. This is of particular interest in RA where an hypoxic synovium is a key feature of disease [216]. Indeed, these data suggest that the hypoxic environment of the joint might favour the generation of Th17 cells rather than Treg and thus contribute to joint damage in RA.

Under acute inflammatory conditions the ability of naïve T cells to sense the environment and differentially activate AhR or HIF-1α for the development of Treg or Th17 may allow an incredibly plastic immune response. Indeed, there is now a consensus that human FOXP3 expressing Treg can simultaneously express RORC, the human homologue of RORγt, and produce IL-17. Moreover, these cells maintained their suppressive function but the provision of a TCR signal in combination with IL-1β, IL-21, IL-23 and IL-2 transformed them into responder T cells [217-219]. In active RA, Treg are defective [220], perhaps in part because they have up-regulated expression of RORC and are producing IL-17. Indeed, ‘ex-Treg’ have been described when $Foxp3^+$ cells are transferred into autoimmune susceptible

mice. These 'ex-Treg' lost expression of Foxp3 and up-regulated production of inflammatory cytokines [221]. Thus, the instability of Treg in inflammatory conditions may contribute to RA pathology.

### 1.2.5 Th22 cells

IL-22 production from CD4 memory T cells was first described in 2000 [222-224]. Much more recently, a distinct subset of T cells producing IL-22 but not IL-17, IFNγ or IL-4 has been identified and named Th22 cells [225-227]. These cells express CCR6, CCR4, CCR10 and can express low levels of RORγt [227, 228], but it is the ligand-dependent transcription factor AhR that is fundamentally required for the production of IL-22 from these cells [229, 230]. Mice deficient in AhR can still produce IL-17, but lose the capacity to produce IL-22 [231]. Plasmacytoid DCs have been shown to drive the differentiation of Th22 cells through the production of IL-6 and TNF[228] and this can be amplified by vitamin D [225]. Moreover, some reports demonstrate a role for IL-23 in the induction of IL-22 production [232, 233] and in mice IL-23 has been shown to be essential for IL-22 production from CD4 T cells [234]. Interestingly, despite a key role for TGF-β in the differentiation of Th17 cells, originally thought to be the primary producers of IL-22 [193], TGF-β has been shown to inhibit IL-22 production [232, 235].

### 1.2.6 The role of monocytes and macrophages during inflammation

During inflammation monocytes are recruited and differentiate into macrophages at the site of inflammation [236]. Monocytes detected in the synovium are activated

and show over-expression of MHC class II, cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines [237]. Despite these activities in the synovium, monocytes are found predominantly in the circulation. It is now clear that monocytes are not simply a population of precursor cells waiting for their call to arms. In fact, a number of monocyte populations exist but it is still unclear if they are functionally different.

The largest human monocyte population has high expression of CD14 (lipopolysaccharide receptor) but lacks expression of CD16 (Fcgamma receptor III). These cells have been shown to be efficient at phagocytosis, and production of cytokines including IL-1 and colony stimulating factors (CSF). A smaller subset of monocytes expresses CD16 in addition to CD14. These cells have been described as less efficient phagocytes, but much better antigen presenters, with increased levels of HLA-DR and high production of IFNα. Furthermore, $CD16^{+}$ monocytes are the major producers of TNF in human blood [238] and bear a resemblance to mature tissue macrophages [239, 240]. Moreover, $CD14^{+}CD16^{+}$ monocytes have been shown to be increased in the periphery of patients with RA compared to healthy controls [241]. A third subset of circulating CD14 low $CD16^{++}$ monocytes represent a population of 'patrolling' monocytes first described in mice. These cells have been shown to be poor phagocytes, exhibiting an anti-inflammatory phenotype in a steady state but specialising in the production of pro-inflammatory cytokines TNF, IL-1β, and CCL3 in response to viruses and nucleic acids [242]. Thus, monocytes may contribute to inflammation outside the synovium and upon reaching the joint readily produce TNF without undergoing differentiation to macrophages.

Macrophage infiltration to the synovium has been shown to correlate with joint damage [243]. This is largely due to the capacity of these cells to drive synovitis via the production of pro-inflammatory mediators. *In vitro* contact between macrophages and FLS is sufficient to drive macrophage production of pro-inflammatory IL-6, GM-CSF and CXCL8 [244]. Similarly, T cells cultured with IL-15 can induce macrophages to make TNF [237] and by contrast IL-10 inhibits the production of pro-inflammatory cytokines from synovial mononuclear cells whilst decreasing expression of HLA-DR [245].

### 1.2.7 The role of B cells in rheumatoid arthritis

T cells assist B cells in the production of antibody. Indeed, the production of autoantibodies is a key feature of RA pathogenesis (discussed in section 1.1.7). Antibodies are produced by plasma B cells, which are somatically mutated B cells with a memory cell phenotype. Plasma cells are specifically identified by flow cytometry via high expression of CD27 and CD38 and low levels of immunoglobulin (Ig) D [246-248]. However, the production of antibody is not the only function of B cells. B cells are also efficient antigen presenting [249] and effector cells and have been shown to be important for the activation of T cells and conversely, the suppression of inflammation.

There is evidence of ongoing T cell help in the RA joint with ectopic follicular units functioning to support autoantibody production [250]. In about 23% of patients $CD20^{+}$ B cells in the rheumatoid synovial tissue can acquire a very distinct architecture similar to that of the germinal centre (GC) of secondary lymphoid tissue.

This structure optimises immune responses through co-localisation of antigen and antigen responsive cells. High levels of CXCL13 from synovial tissue cells and lymphotoxin beta (LT-β) from B cells can predict the emergence of germinal centres in patients suggesting that B cells are at least partly responsible for the formation of these GC [251]. Adoptive transfer of $CD4^+$ T cell clones into a SCID mouse transplanted with human synovium drove the production of IFNγ, TNF and IL-1β. T cell activation upon transfer could be abrogated by the elimination of $CD20^+$ B cells and the elimination of GC in the joint. This suggests that T cell activation requires B cell interaction in the GC. Importantly, this capacity of B cells could not be replaced by other APC and so this indicates that the interaction of B cells with T cells has a key role in T cell activation [252].

Despite a capacity to drive inflammation, B cell deficient mice develop exacerbated EAE suggesting a protective role for B cells [253]. B cells from the spleen of CIA mice stimulated with anti-CD40 were shown to become IL-10 producers. Transfer of these cells back into CIA mice prevented disease whilst B cells from IL-10 knockout mice failed to have this effect [254]. Moreover, IL-10 producing cells were shown to be a distinct B cell subset in mice. Transfer of the transitional-2-marginal zone precursor B cells, present in the spleen of mice under normal conditions but increased in the remission phase of arthritis, ameliorated CIA [255]. Recently a population of regulatory B cells has been described in humans. Identified as $CD19^+CD24^{hi}CD38^{hi}$, upon stimulation with CD40, these cells suppressed the differentiation of Th1 cells through production of IL-10. This capacity was reversed by blockade of CD80 and CD86 suggesting that the effect mediated by these B cells involves offering co-stimulation to T cells in addition to their production of IL-10

[256]. An interesting recent development has shown that mice that specifically lack IL-10-producing B cells have a reduction in $Foxp3^+$ regulatory T cells and a concomitant increase in inflammatory cytokines suggesting that these two regulatory subsets may have a developmental relationship [257].

### 1.2.8 Tumour necrosis factor

TNF forms a membrane bound homotrimer (mTNF) that can be cleaved by TNF-α converting enzyme to generate a soluble molecule (sTNF). Both soluble and mTNF can bind and signal to TNF receptors (TNFR). TNFR1 (p55) is ubiquitously expressed and is thought to favour signals via soluble TNF. The cytoplasmic domain of TNFR1 contains a death domain important in mediating apoptosis in response to TNF signals [258]. Animals lacking TNF or TNFR1, demonstrated that TNFR signalling was vital for the formation of B cell follicles in secondary lymphoid organs but had little effect on humoral immunity [259]. TNFR2 (p75) expression is limited to the immune system and is thought to preferentially transduce signals mediated by mTNF [260] and lymphotoxin family members. Furthermore, TNFR2 mediates lymphocyte proliferation and apoptosis of mature activated lymphocytes in response to TNF [258]. Both TNF receptors are widely expressed on synovial tissue, largely out-weighing production of TNF [261].

Most members of the TNFR family activate the transcription factor NFκB (Figure 1.2). In unstimulated cells NFκB is present in the cytoplasm bound to IκB to prevent it entering the nucleus. This ready state of NFκB allows rapid signalling in response to cellular activation, a vital characteristic of the immune system. Upon stimulation,

IκB is phosphorylated by the IKK (IκBα kinase) complex. IKK catalyses the degradation of IκB allowing NFκB to translocate to the nucleus (Figure 1.5). A wide range of genes have NFκB-binding domains in their promoter regions and these include pro-inflammatory cytokines, chemokines, immune receptors and adhesion molecules, which enhance immune response [262].

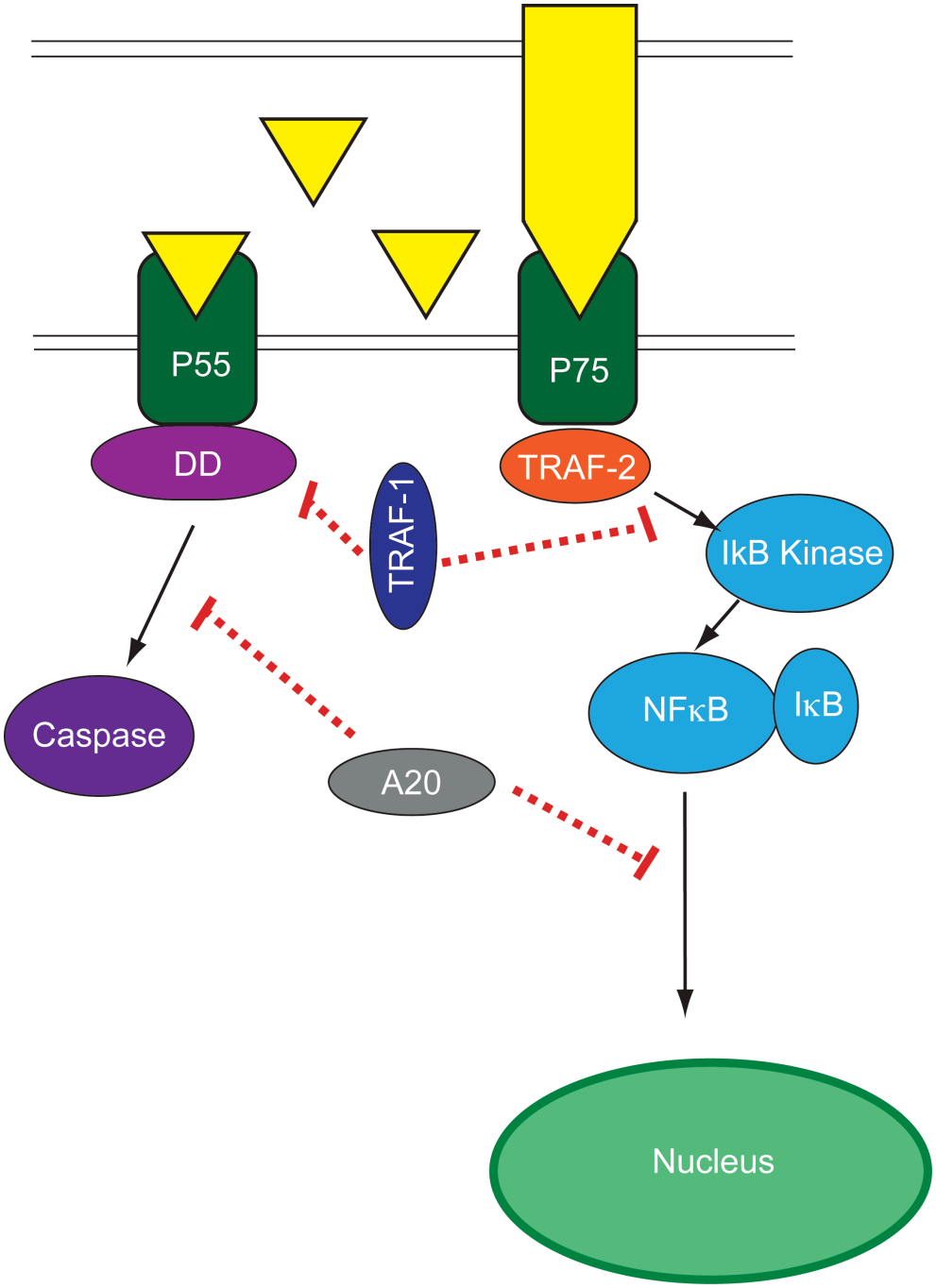


**Figure 1.5. NFκB signalling pathway**

The binding of soluble TNF to TNF receptor (TNFR) 1 can mediate apoptosis via associated death domains (DD) signalling to caspases. Signalling of membrane TNF through TNFR2 activates IκB Kinase (IKK). In turn this leads to the degradation of IκB, allowing NFκB to translocate to the nucleus where it can directly bind to many genes, including those that control inflammation. Both of these signals can be negatively regulated by A20 or TRAF-1. Thus the association of SNPs in these genes with RA heavily implicates differential responsiveness to TNF with development of disease.

TNF is a pluripotent molecule that sits at the pinnacle of inflammation. The association of SNPs in the TNF receptor signalling pathway (see section 1.1.6.1) with the development of RA implicates TNF as a trigger of autoimmunity. Moreover, the high levels of TNF detected in the joints of patients with RA [263] indicates that it continues to modulate the immune system throughout disease pathogenesis.

In RA, macrophage-derived TNF has been shown to drive angiogenesis contributing to the aberrant disorganised vascularisation of the synovium [264]. Moreover TNF can act on endothelial cells to induce the expression of adhesion molecules that promote the recruitment of circulating immune cells to the joint [265]. Stimulation of synovium-resident cells such as fibroblast-like synovial (FLS) cells or chondrocytes with TNF can stimulate the production of matrix degrading enzymes [266], chemokines and proinflammatory cytokines which can in turn contribute to further joint damage and cell recruitment [267]. TNF can also drive the maturation of dendritic cells in the RA synovium stabilising MHC expression and upregulating co-stimulatory markers to activate T cells [268]. Furthermore, many of the T cells in the rheumatoid synovium share phenotypic characteristics with T cells activated by cytokines *in vitro*, high levels of TNF contributing to this phenotype. In turn these cells have been shown to stimulate TNF production from monocytes in a contact-dependent manner [269], initiating a positive feedback system which drives TNF production in the synovium.

The infiltration of TNF-producing macrophages to the RA synovium is directly correlated with joint damage [243]. Indeed, TNF can contribute to the dysregulation in bone metabolism associated with cartilage destruction in RA. The binding of

RANK (receptor activator of nuclear factor kappa-B) on monocytes to its ligand RANKL on FLS or T cells drives monocyte differentiation to cartilage degrading osteoclasts [270]. TNF can bind and signal to osteoclast precursors via their expression of TNFR1 [271] substantially reducing the amount of RANKL required for the differentiation of osteoclasts [272]. Moreover, this activity of TNF can increase the number of osteoclast precursors available for differentiation. In patients with TNF driven diseases this provides an additional link between high concentrations of TNF and cartilage degradation [273]. In mice with transgenic over-expression of TNF, osteoclastogenesis was inhibited by treatment with an osteoclast inhibitory factor, osteoprotegerin (OPG), though there was no improvement in paw swelling in these animals [274]. Thus in RA, TNF modulates distinct pathways of inflammation and cartilage degradation. This highlights the benefits of targeting TNF for therapeutic purposes.

### 1.2.9 IL-17

IL-17 is produced primarily by Th17 cells and a small subset of innate lymphocytes named γδ T cells. By contrast, IL-17 receptor expression is widespread in the immune system [275]. One of the primary roles of IL-17 is in the recruitment of neutrophils to the site of inflammation [276]. In human synoviocytes this is dependent on NFκB signalling and the production of CXCL8 [277]. In moderate inflammation, one billion neutrophils per day enter the RA joint, but due to lack of egress, stay there mediating wide scale non-specific destruction of the synovial tissue. Neutrophils are rapidly activated by debris and immune complexes and degranulate releasing proteases, prostaglandins and reactive oxygen intermediates

(ROI) which contribute to tissue destruction [278]. Additionally, ROI and granular enzymes from neutrophils can contribute to the breakdown of hyaluronic acid, which impairs viscosity and lubricative potential of the synovial fluid [279].

In addition to contributing to joint damage through the recruitment of neutrophils, IL-17 is also a potent stimulator of osteoclastogenesis [280]. Adenoviral driven over-expression of IL-17 in CIA promoted osteoclastogenesis, bone destruction and expression of RANK/RANKL in the synovial infiltrate and at sites of erosion. This increased expression of IL-17 was associated with an increased ratio of RANKL to OPG, compared to non-transfected animals with a similar clinical score. Furthermore, systemic OPG treatment of the IL-17 transfected mice was able to prevent joint destruction through restoration of this balance [281].

Studies in mice have provided substantial insight into the role of IL-17 in autoimmune inflammation. Overexpression of IL-17A in the joints of mice with CIA increased the recruitment of neutrophils to the joint and exacerbated disease [282]. Furthermore CIA was shown to be ameliorated by treating animals with anti-IL-17 [283]. In SKG mice that spontaneously develop an autoimmune arthritis, pathology is driven by a cytokine milieu that favours the production of Th17 cells. Moreover, there is no evidence of arthritis in IL-6 or IL-17 SKG deficient mice but development of disease is exacerbated in IFNγ knockout animals due to an increase in Th17 cells [284]. These data suggest that self-reactive T cells which escape the thymus in RA-susceptible individuals may be pre-disposed to becoming Th17 cells that can, in turn, promote an inflammatory environment that favours their stability and differentiation.

Similar to many inflammatory cytokines, IL-17 can drive the production of cytokines and chemokines from other immune cells. Indeed, IL-17 produced by T cells in the joint can stimulate macrophages to produce IL-1 and TNF [285] and synergise with TNF or IL-1 to enhance the production of CXCL8 or G-CSF to levels above that induced by IL-17 alone [286]. Like TNF, IL-17 can contribute to the reduction in apoptosis observed in RA through the induction of synoviolin [287], a negative regulator of the pro-apoptotic molecule p53 [288].

### 1.2.10 IL-22

IL-22 is a member of the IL-10 family of cytokines and in addition to helper T cells,IL-22 is produced by a subset of CD8 memory T cells, γδ T cells, NK cells and innate lymphoid cells [225, 289]. IL-22 signalling is mediated via a heterodimeric receptor made up of the IL-22R and the IL-10Rβ[223, 224]. Expression of this receptor complex is predominantly associated with epithelial cells [290]. The absence of IL-22R expression on cells of the immune system means that unlike most cytokines, IL-22 does not signal to immune cells [290]. Downstream of its receptor, IL-22 activates STAT3 [224, 290-294]. Indeed, IL-22 has been shown to activate STAT3 more strongly than IL-6, the prototype STAT3 activating cytokine [295]. IL-22 mediates keratinocyte anti-microbial defence, migratory capacity and terminal differentiation [296, 297] in part through the induction of IL-20 which amplifies these responses [298]. The effects of IL-22 are further amplified by TNF [299].

In disease, IL-22 has been shown to be important for epithelial remodelling and driving keratinocyte proliferation and epithelial hyperplasia due to the expression of IL-22R on keratinocytes [297, 299]. The role of IL-22 at epithelial barriers is

highlighted by mice with over-expression of IL-22 which develop a skin disease closely resembling psoriasis [299]. Indeed, IL-22 has been shown to be associated with increased disease severity in patients with psoriasis [290, 296, 300]. *In vitro* it has been shown that IL-22 promotes osteoclastogenesis, suggesting a pro-inflammatory role for IL-22 in arthritis. Indeed, IL-22 deficient mice are less susceptible to CIA and demonstrate decreased pannus formation than control mice [301]. In patients with RA, IL-22 levels are increased in the synovium and the blood [208, 302-304]. Mirroring the murine models, IL-22 has been shown to promote osteoclastogenesis through the induction of RANKL on human synovial fibroblasts [305]. Moreover, serum IL-22 levels have been shown to correlate with radiographic progression in RA [306].

In contrast, IL-22 has been shown to be protective in hepatitis [307, 308], IBD [291, 309], graft versus host disease in heart allografts [310], autoimmune myocarditis [311], allergic airway inflammation [312] and uveitis [313]. Furthermore, the presence of IL-22 producing cells has been associated with tumour progression and a worse clinical prognosis [233, 314]. The role of IL-22 as a mediator of tissue repair rather than destruction has been shown in the delayed healing of mucosa in IL-22 deficient mice [293], the involvement of IL-22 in regeneration of liver tissue [294] and the IL-22 driven repair of keratinocytes after wounding [226]. Furthermore IL-22 may mediate the prevention of systemic inflammation driven by lipopolysaccharide (LPS) through the induction of hepatic LPS binding protein [315].

The pro versus anti-inflammatory capacity of IL-22 has previously been investigated using an airway inflammation model. The authors showed that IL-22 deficient mice and mice treated with an IL-22 neutralising antibody had ameliorated disease. However, if the same disease was induced in IL-17 deficient mice, the neutralisation of IL-22 exacerbated disease. Furthermore, whilst *in vitro* administration of IL-22 protected airway epithelial cells from apoptosis, co-administration of IL-17 worsened airway inflammation. Thus the authors proposed an interesting model whereby the presence of IL-17 was the deciding factor in the pro- versus anti-inflammatory properties of IL-22 [316].

### 1.2.11 IL-6

IL-6 is a pleiotropic cytokine first identified as a B cell differentiation factor [317] required for the differentiation of plasma cells [318] and promoting B cell antibody production [319]. It is now known that IL-6 can also drive the production of acute phase proteins [320], bone metabolism [321], angiogenesis [322], nerve growth [323, 324], the production of glucocorticoids [146] and the differentiation of Th17 cells [188-191]. Moreover, IL-6 is particularly important in tipping the balance of monocyte differentiation towards macrophages rather than DCs [325, 326].

IL-6 is produced by many immune cells as well as fibroblasts, keratinocytes and endothelial cells [327]. It is part of a family of cytokines that utilise two signalling receptors, an IL-6 receptor (IL-6R) and a signal transducing molecule gp130. Upon binding of IL-6 to its membrane bound receptor, gp130 homodimerises and binds the IL-6/IL-6R complex, forming a functional receptor. IL-6R expression is limited to

monocytes, neutrophils, macrophages, hepatocytes and some lymphocytes, which is somewhat at odds with the broad spectrum of IL-6 activity. However, in addition to being a membrane bound molecule, IL-6R can be cleaved from the surface of cells or generated by alternatively splicing mRNA to form a soluble molecule. Due to the distinctive signalling mechanism of IL-6, soluble IL-6R can bind IL-6 and signal to any cell bearing expression of gp130, termed trans-signalling. Furthermore, gp130 is expressed on most body tissues, which explains the pleiotropic function of IL-6. As with many cytokines, expression of IL-6 can be modulated by a soluble receptor: in this case soluble gp130 can prevent the binding of the IL-6/IL-6R complex to gp130 on the cell membrane [328].

IL-6 deletion protects mice from developing CIA and also a second murine arthritis model, antigen-induced arthritis (AIA) [329, 330]. Notably, mice deficient in IL-6 supplemented with recombinant IL-6 did not have a restoration of wildtype AIA severity. Only when an IL-6/IL-6R construct was transferred did disease activity return to levels of a wildtype mouse. Moreover, blocking sIL-6R signalling in wildtype mice using a soluble gp130 molecule ameliorated disease [331]. Thus, the induction of arthritis appears to specifically require IL-6 trans-signalling. Indeed, IL-6 activities specifically mediated via trans-signalling have been shown to be important for leukocyte recruitment and activation in RA [332].

There are high levels of IL-6 in the synovial fluid of patients with RA [333]. This is associated with increased sIL-6R levels compared to patients with OA [331, 334] and is correlated with the presence of osteoclast precursor cells [334]. FLS produce IL-6 when stimulated with IL-1, TNF or IL-17 and IL-6/sIL-6R is sufficient to

induce proliferation and RANKL expression on FLS [335, 336]. Furthermore, IL-6 can rescue T cells from apoptosis via the activation of STAT3 [337-339].

### 1.2.12 The effects of inflammation on regulatory T cells

In an inflammatory environment, such as that observed with RA, the full function of Treg may be inhibited by inflammatory cytokines. Indeed, IL-6 can make responder T cells resistant to Treg suppression [340] and TNF has been shown to impair the expression of FOXP3 [130]. Recently however, the relationship between inflammation and Treg has been readdressed with data suggesting that inflammatory signals may be important for the induction of Treg. Indeed, TNF was shown to drive the expansion and activation of Treg via TNFRII [341], which is widely expressed on Treg [130]. Moreover, mice in which overexpression of human TNF drives an arthritic pathology, develop a more severe form of arthritis in the absence of global TNFR2 expression [342] or TNFR2 expression on haematopoetic cells [343]. There is a well-documented increase in $FOXP3^+$ cells in the synovial fluid of patients with active RA and these cells are potent suppressors in *in vitro* suppression assays. Though the activated T cells from this site appear to be resistant to suppression [127]. Thus, perhaps cells can be induced by inflammation, but the presence of inflammation makes responder T cells resistant to suppression. As soon as inflammation begins to attenuate, iTreg can begin to function to suppress disease.

### 1.2.13 Synovitis

Upon immune response to self-antigen in the joint, positive feedback leads to the accumulation of cells in the joint, and the promotion of factors which drive bone and cartilage erosion, ligament damage and bone marrow inflammation [267]. In addition to these features of disease, inflammatory cytokines have been proposed to contribute to the fatigue and malaise associated with RA [344]. Whilst morning stiffness and warm joints are explained by increased extracelluar fluid around the inflamed area and dilation of the synovial blood vessels deep in the tissue.

Inflammation of the synovium is associated with increased vascularisation, cellular recruitment and production of cytokines. A pannus of proliferating tissue grows over the cartilage of the joint and becomes heavily infiltrated with inflammatory, degradative enzyme producing cells. The pannus infiltrate includes large numbers of macrophages that produce enzymes which can degrade cartilage matrix [345]. Furthermore, many cells resident in the synovium contribute to tissue damage in RA, including chondrocytes, osteoclasts and fibroblast–like synovicytes (Figure 1.6).

A surprisingly small amount of damage to articular cartilage is required before joints are weakened to the point where daily activities or exercise on weight bearing joints leads to disintegration [14]. Bone erosion occurs in about 75% of RA patients within 2 years of diagnosis [346].

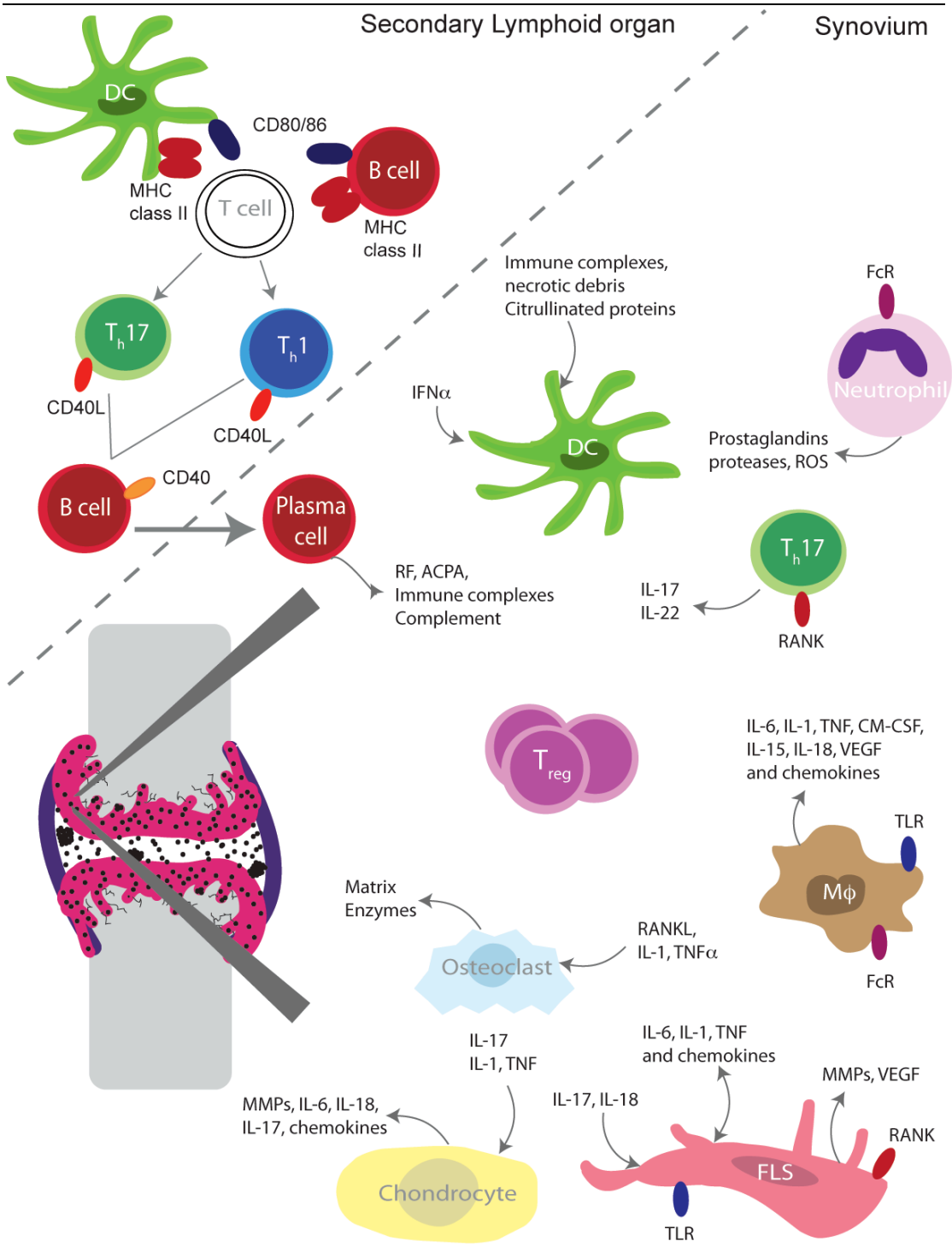


**Figure 1.6. Inflammation in the rheumatoid joint**
In the secondary lymphoid organ antigen is presented to T cells. This drives the maturation of B cells to plasma cells. Upon migration to the joint, these produce autoantibodies including rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) contributing to the formation of immune complexes. Antibodies can also activate infiltrating macrophages (mf) and neutrophils via their Fc receptor (FcR) and can initiate phagocytosis by dendritic cells (DC). Toll-like receptors (TLR), expressed on mf and fibroblast-like synovial cells (FLS) can recognise bacterial peptides. In response to stimulation, cells of the synovium can produce pro-inflammatory cytokines that can in turn stimulate the production of cytokines, chemokines and matrix metalloproteinases contributing to joint damage. Functional regulatory T cells are also found in the joint but responder T cells from this site are resistant to suppression (adapted from [267]).

#### *1.2.13.1 Angiogenesis*

Hypoxia is a potent stimulator of angiogenesis and the inflamed joint is extremely hypoxic compared to healthy controls [216]. The synovial lining has dense microvasculature to provide nutrients to the avascular cartilage, but in RA there is a redistribution of blood vessels and an increased vasculature deep in the synovium. This new vasculature is less densely arranged, generating pockets of hypoxia [216, 347]. Adenoviral gene transfer of vascular endothelial growth factor (VEGF) receptor to block angiogenic signals, reduced the severity of CIA and associated joint damage [348], suggesting that vascularisation of the synovium is key to RA pathogenesis. The increased vascularisation observed in RA is concomitant with insufficient lymphangiogenesis, resulting in an accumulation of immune cells as the joint.

#### *1.2.13.2 Homing of cells to the joint*

Inflammatory cytokines such as TNF and IL-1 can act on endothelial cells to induce the expression of adhesion molecules (including integrins and selectins) that promote the recruitment of cells to the joint. In the synovium, fibroblast-like synovicytes, monocytes and macrophages are the major producers of chemokines, which act to attract inflammatory cells to the joint [14, 267, 349] (Table 1.3, pg. 86).

**Table 1.3 Chemokines involved in rheumatoid arthritis pathogenesis.**

| Chemokine | Receptor(s) | Recruits |
|---|---|---|
| CXCL8 (IL-8) | CXCR1<br>CXCR2 | Myeloid cells |
| CXCL5 | CXCR1<br>CXCR2 | Myeloid cells |
| CCL2 (monocyte chemoattractant protein-1) | CCR2<br>CCR11 | Monocytes, DC, basophils and polymorphonuclear cells |
| CCL5 | CCR1<br>CCR3<br>CCR5 | Memory T cells, granulocytes, Th1 cells, macrophages, monocytes, DC |
| CCL3 (macrophage inflammatory protein-1) | CCR1<br>CCR5 | Memory T cells, Th1 cells, monocytes, macrophages, DC |

#### *1.2.13.3 Matrix enzymes*

Cartilage degradation during RA is driven by over production of matrix metalloproteinases (MMP)s, proteolytic enzymes that have the capacity to degrade proteins of the extracellular matrix. MMPs are divided into subgroups including: collagenases, gelatinases, stromelysins and membrane-type MMPs. MMPs are secreted as proenzymes and activated by serine proteases such as plasmin or elastase, or by other MMPs including MMP-3 and MT1-MMP. In rheumatoid synovial tissue, increased amounts of MMPs are expressed, but certain MMPs have been identified at the site of cartilage degradation in patients with RA [350]. The function of these is outlined in the table below (Table 1.4, pg 87). Indeed, inhibition of MMP-1 production in SCID mice injected with RA synovial tissue resulted in reduced

invasiveness of FLS [351]. Moreover, over-expression of MMP antagonists, tissue inhibitors of metalloproteinases (TIMPs), resulted in the inhibition of FLS-mediated invasiveness and cartilage destruction in the same synovial transplant model [352]

**Table 1.4 Matrix metalloproteinases associated with RA**

| MMP | Type of enzyme | Other functions |
|---|---|---|
| MMP-1 | Collagenase I | Cell migration |
| MMP-3 | Stromelysin | Cell migration |
| MMP-9 | Gelatinase | Chondrocyte apoptosis and recruitment of osteoclasts |
| MMP-13 | Collagenase III | Osteoclast activation |

## 1.3 Therapy for rheumatoid arthritis

Initial treatment for RA consists of disease modifying anti-rheumatic drugs (DMARDS) such as methotrexate, sulfalsalazine, hydroxychloroquine, prednisolone or leflunomide. The most commonly used DMARD is methotrexate (MTX), an anti-metabolite drug that was initially thought to work by inhibiting cellular proliferation. It is now known that methotrexate increases the apoptosis of T cells, increases endogenous adenosine release, alters the expression of adhesion molecules, influences production of IL-1 and IL-2 and directs bone formation [353]. Aggressive treatment with a combination of DMARDs soon after diagnosis has been shown to be highly beneficial for the control of inflammation and bone erosion in RA [354-356].

### 1.3.1 Biologic Therapy

Recently there has been a shift towards a more targeted approach - in particular, biologic therapies that target pathways known to be important in the pathogenesis of autoimmune disease (Figure 1.7). Principally due to cost considerations a combination of conventional DMARDs is used first but if these are inadequate, anti-TNF therapy is indicated. In addition, for patients who do not respond to anti-TNF therapy or who cannot take this medication for other reasons (e.g. previous malignancy) the anti-B cell depleting agent, rituximab, the anti-IL-6 receptor, tocilizumab and the CTLA-4 Ig, abatacept are also available [357].

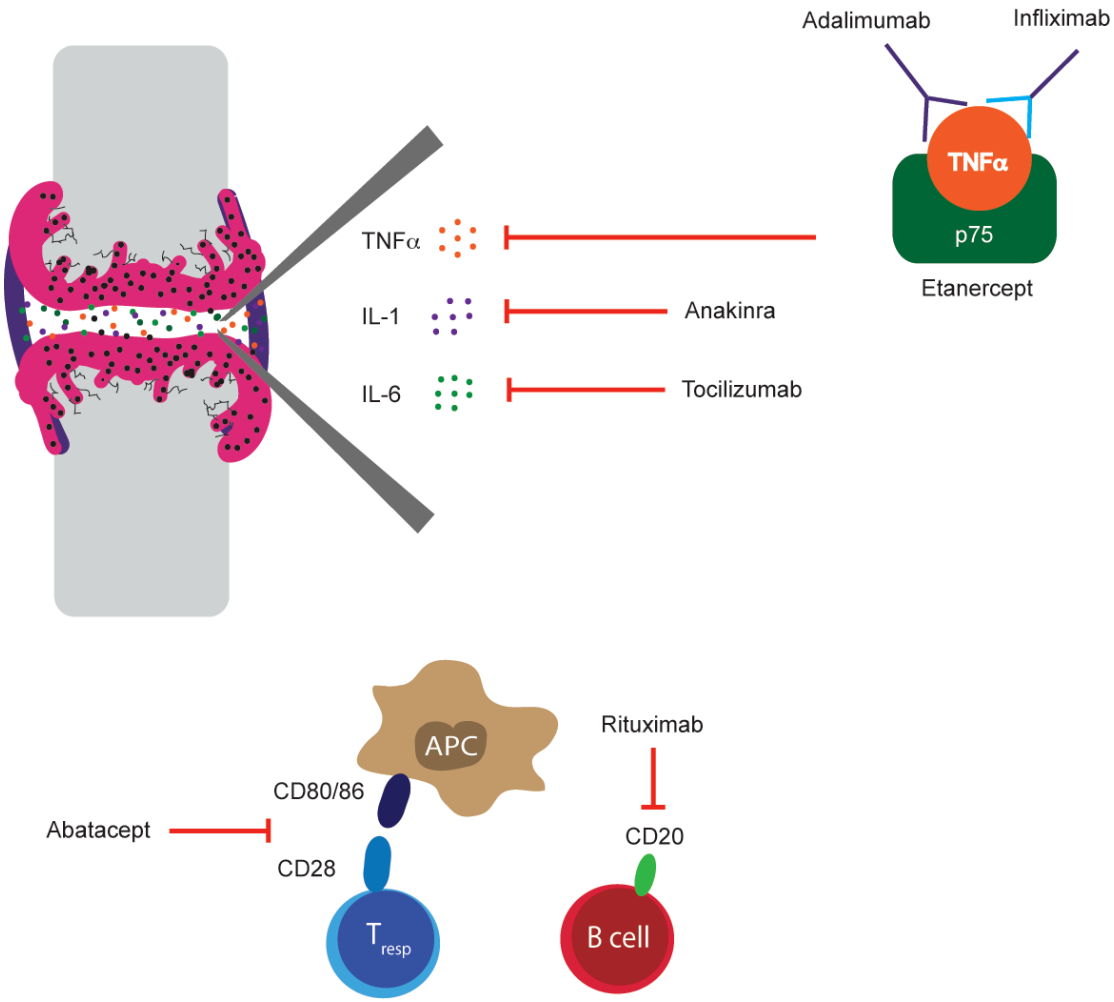


**Figure 1.7. Biologic therapies:**
Biologic therapies in RA have targeted pro-inflammatory cytokines with the anti-IL-1 receptor, anakinra, the anti-IL-6 receptor, tocilizumab and the anti-TNF therapies. A number of anti-TNF therapies are available to treat RA. These include the soluble TNFR2 molecule etanercept, the chimeric mouse/human anti-TNFα monoclonal antibody, infliximab and the entirely humanised anti-TNFα antibody, adalimumab. Biologic therapies have also been used to block cell-cell interactions. The CTLA-4 immunoglobulin prevents the co-stimulatory activity of CD80 and CD86 on antigen presenting cells (APC). Depleting B cells using an anti-CD20 antibody, rituximab, has also been shown to be an effective therapy for RA.

### 1.3.2 Anti-Tumour Necrosis Factor therapies

Initial investigations in mice showed that the blockade of TNF in autoimmune models such as CIA ameliorated disease [358]. Later that it was shown that anti-TNF was an effective therapy for RA [359]. There are currently a number of anti-TNF therapies available. These include: etanercept, comprisingthe extracellular portions of TNFR2 fused to the Fc portion of IgG1; infliximab, a mouse/human chimeric monoclonal anti-TNF IgG1 antibody and adalimumab, a human monoclonal IgG1 antibody. Etanercept efficiently binds trimers of TNF, but unlike the monoclonal antibodies, also binds the closely related LTβ. In addition, patients can be prescribed a second humanised monoclonal antibody, golimumab and certolizumab pegol, a PEGylated Fab' fragment of a humanised anti-TNF monoclonal antibody. Patients at University College Hospital (UCH) are predominantly described infliximab, adalimumab and etanercept so golimumab and certolizumab pegol are not discussed further.

Infliximab is administered by intravenous infusion every 4 to 6 weeks and has a terminal half-life of 8-10 days. Due to its chimerism, infliximab is more immunogenic than the humanised molecules and must administered alongside an additional immunosuppressant such as methotrexate. Etanercept is administered subcutaneously via a pre-filled ‘pen’ device taken at home once or twice a week and has a half-life of approximately 4 days. Adalimumab is also administered sub-cutaneously every 2 weeks but has a much longer half-life of 10-20 days [265, 360]. Infliximab is now less often prescribed as patients prefer subcutaneous injections to infusions. Whilst all three therapies reduce TNF and are effective in the treatment of RA, clinical differences between the therapies have been noted. Monoclonal

antibody therapy, but not etanercept therapy, is effective in granulomatous diseases such as Crohn's disease [361, 362] and sarcoidosis [363, 364]. On the other hand, RA patients treated with either adalimumab or infliximab have a 7-17 fold higher risk of developing a *Mycobacterium tuberculosis* (TB) infection compared to RA patients treated with etanercept [365].

Initial studies investigating the differences observed between the monoclonal anti-TNF antibody (infliximab) and the soluble TNFR2 (etanercept) found that etanercept could only transiently bind trimers of TNF. Furthermore the TNF molecules released by dissociation with etanercept were shown to be biologically active. In contrast, infliximab could bind both monomers and trimers of TNF, forming stable complexes with each molecule. Both infliximab and etanercept have been shown to bind to membrane TNF and were both able to prevent the ability of mTNF to activate human endothelial cells. However, infliximab binding to mTNF was more effective in blocking the activation of endothelial cells than etanercept. Furthermore, infliximab binding of mTNF was more stable than etanercept with more infliximab molecules binding to mTNF with a higher avidity. This led the authors to conclude that infliximab mediates a more complete and sustained neutralisation of TNF than etanercept [366].

### 1.3.3 Anti-TNF therapy for the treatment of rheumatoid arthritis

All anti-TNF therapies are recommended for the treatment of RA patients who have failed methotrexate and at least one other DMARD and have been classified as active with a DAS >5.1 by a rheumatologist on at least two occasions (NICE guidelines

October 2007). In RA, approximately 40% of patients respond to anti-TNF therapies with at least 50% improvement in DAS-28 score. Furthermore, the observed changes after anti-TNF have provided novel insight into the roles of TNF in the pathogenesis of RA

In a subset of patients, treatment with anti-TNF therapies results in long lasting responses associated with prevention of articular damage. Indeed, patients treated with anti-TNF antibody showed a reduction in synovial infiltration of granulocytes, macrophages, B cells and T cells and this was shown to correspond with a drop in serum levels of CCL2 and CXCL8 [367]. Treatment with etanercept was shown to result in a drop in MMP-1 and MMP-3 as well as a reduction in the ratio of MMPs to TIMP-1 [368]. Furthermore, long-term therapy with adalimumab decreased the levels of soluble intercellular adhesion molecule (ICAM) and MMPs and demonstrated a positive effect on radiological outcome [369]. *In vitro* treatment of synoviocytes with infliximab was shown to inhibit RANKL expression and increase expression of OPG in the supernatant [370]. In patients, treatment with infliximab or etanercept for 8 weeks increased the expression of OPG in synovial tissue, but had no effect on the RANKL expression. However, therapy was shown to reduce the ratio of RANKL:OPG, which would contribute to a reduced osteoclastogenesis and thus reduced cartilage damage [371].

Anti-TNF therapies also appear to alter autoantibody titres in responding patients. Indeed, it has been suggested by one study that patients responding to anti-TNF have a decrease in serum titres of rheumatoid factor and ACPA [372]. Though, there is some controversy. A second study claims that only RF levels are reduced, whilst

ACPA levels remain the same [373]. A third study shows a reduction in both sets of antibodies, but only in responders and in this too RF was more strongly affected than ACPA [374]. Thus, all 3 studies agree that there is a fall in RF antibodies, which is perhaps unsurprising given the link between inflammation and the presence of RF antibodies (see section 1.1.7.2). More studies will have to be done to elucidate what, if any, effect the blockade of TNF has on ACPA production.

The synovial synthesis of TNF was reduced after infliximab therapy and this was associated with a fall in IL-1α [375], reduced levels of IL-6 and acute proteins such as amyloid A, haptoglobin and fibrinogen [376 ]. Furthermore, angiogenic factor VEGF was significantly decreased in the serum of anti-TNF antibody treated patients with RA [377], highlighting the role of TNF in driving aberrant angiogenesis in the RA synovium. An integral role for TNF in the resistance of cells to apoptosis in RA was confirmed by an induction of macrophage apoptosis *in vivo* and *in vitro* in RA synovial biopsies of patients on long term treatment with both etanercept and infliximab [378].

Thus anti-TNF therapy targets some of the most aggressive aspects of RA resulting in a clinical response that reduces pain and disability.

### 1.3.4 Effects of anti-TNF therapy on regulatory T cells

Inflammation is known to negatively modulate Treg function. Given the abundance of TNF in the synovium of patients with RA it was proposed that the response to anti-TNF therapy might be mediated, in part, via a reversal of the Treg defect

observed in these patients. Indeed, Treg from RA patients treated with infliximab were shown to suppress IFNγ and TNF at comparable levels to healthy controls. Moreover, it was found that these patients had an increase in the number of peripheral Treg [124]. This heavily implicated TNF in the modulation of Treg number and function. Surprisingly, suppression by Treg from patients treated with infliximab was not mediated via a restoration of CTLA-4 expression on Treg, as levels of CTLA-4 were similar to patients with active RA [37].

This restoration of suppressive function by infliximab therapy in the absence of a restoration of CTLA-4 expression, led to an investigation of the phenotypic and functional characteristics of the Treg from these patients. *Ex vivo* staining revealed that patients treated with infliximab had increased levels of $CD62L^-$ Treg compared to healthy controls and patients with active RA [86]. Comparison of the suppressive function of $CD62L^+$ and $CD62L^-$ Treg showed unsurprisingly, that both were defective in patients with RA. However, whilst $CD62L^+$ Treg were the most potent suppressors in healthy controls, in infliximab treated patients there was a distinct shift. The suppressive ability of $CD62L^+$ Treg in infliximab treated patients resembled that of patients with active RA but $CD62L^-$ Treg were much more potent suppressors than the same population in healthy controls or patients with active RA. Moreover, in contrast to functional Treg in healthy controls, the neutralisation of TGF-β and IL-10 abrogated the suppressive ability of $CD62L^-$ Treg. Furthermore, it was shown that the *in vitro* addition of infliximab to purified $CD25^-$ cells from patients with RA, but not healthy controls, induced a population of $FOXP3^+$ cells. These cells were shown to be $CD62L^-$ and examination of their function *in vitro* found that they could suppress IFNγ. Thus, it was concluded that infliximab induced

Treg with the capacity to suppress IFNγ rather than restoring the suppressive ability of existing Treg [86].

### 1.3.5 Anti-IL-6 receptor (tocilizumab)

The targeting of IL-6 with an anti-IL-6 receptor was first suggested to be an effective therapy in mice, where treatment was sufficient to suppress CIA and protect joints from destruction [379]. Tocilizumab is a humanised anti-human IL-6 receptor antibody of the IgG1 subclass, shown to compete for both membrane bound and soluble receptors of IL-6. It is administered by infusion once every 4 weeks and has a half life of approximately 10 days [380]. Tocilizumab has been shown to be a successful therapy in the treatment of RA refractory to both traditional DMARDS [381] and anti-TNF [382]. Tocilizumab in combination with methotrexate therapy is indicated if patients have moderate to severe active RA and have failed at least one anti-TNF therapy and rituximab (NICE guidelines August 2010). More recently, NICE approved tocilizumab as a first line biologic agent (NICE guidelines February 2012).

Compared to methotrexate therapy, tocilizumab was shown to reduce proliferation, beta-1 integrin expression and $CD20^+$ cells in the joint. In contrast, levels of TNF, CD68 and MMP-3 were unchanged in tocilizumab treated patients 6-8 months after therapy [383]. Highlighting the importance of IL-6 in B cell antibody production, another study showed that in tocilizumab treated patients there was a fall in the number of memory B cells, IgA and IgG expressing B cells and a drop in serum immunoglobulin levels between weeks 12-24 of therapy [384]. Tocilizumab has also

been shown to inhibit joint damage, suggesting that IL-6 contributes to the destruction of cartilage in RA [385].

#### *1.3.5.1 Anti-CD20 (rituximab)*

Rituximab targets and transiently depletes $CD20^+$ B cells, which include pre-B cells and mature B cells but does not directly deplete autoantibody producing plasma cells. However, short-lived plasma cells are depleted and autoantibody titres do fall. Despite this, patients depleted of B cells demonstrate a clinical response including a reduction in joint erosion [386]. The improvement in disease despite the presence of antibodies highlights the importance of other B cell functions in RA such as antigen presentation, the production of cytokines and the formation of ectopic germinal centres.

#### *1.3.5.2 CTLA-4 Ig (abatacept)*

Abatacept (CTLA-4 Ig) acts as an exogenous CTLA-4 molecule that blocks the interaction between CD28 and CD80/86. A recent study has shown by histochemical staining that whilst levels of TNF, IL-6 and VEGF were unchanged in the synovium of RA patients treated with abatacept, there was a reduction in MMP-3, CD68, CD4, CD8, CD20, CD80 and CD86 [387], suggesting that abatacept can reduce cellular recruitment to the synovium. Whether this is a direct effect of abatacept therapy or an indirect effect through a reduction of the capacity of APC to offer co-stimulation to T cells remains to be established. Furthermore, it has been shown that binding of CTLA-4 to monocytes inhibits RANKL expression and TNF-induced osteoclastogenesis *in vitro* in the absence of T cells [388]. Thus, treatment with

abatacept has the potential to prevent further joint erosion. Notably, many cells in the synovium up-regulate MHC molecules during inflammation [389]. Blockade of co-stimulatory molecules with CTLA-4 Ig may prevent the ability of these MHC molecules to successfully activate T cells and perhaps offers an additional explanation as to why therapy with abatacept is effective.

#### *1.3.5.3 IL-1 receptor antagonist (anakinra)*

Like disease itself, response of patients to immune targeted biologic therapies provides insight to disease pathogenesis. Whilst the therapies described above have all shown to be effective therapies for RA, anakinra, an IL-1 receptor antagonist that neutralises the activity of IL-1 is not a particularly effective therapy in RA. This was quite an unexpected finding, since IL-1 is widely expressed in the human synovial membrane in patients with RA [390] and blocking IL-1 in mouse models of RA resulted in reduced inflammation and substantial reduction in joint damage [391, 392]. Moreover, crossing human TNF transgenic mice to IL-1α and IL-1β knockout mice, did not prevent synovitis but did prevent erosion of cartilage, identifying IL-1 as a key mediator of bone erosion in inflammatory arthritis [393]. However, whilst therapy with anakinra in patients with RA had some effect on inflammation and joint erosion, in neither of these aspects was anakinra a more effective therapy than anti-TNF [394, 395]. Notably, the same doses of anakinra used to treat patients with RA provided an effective therapy for systemic-onset juvenile idiopathic arthritis and adult-onset Still's disease [395]. Thus IL-1 may not be as important in the pathogenesis of RA as initially suggested by animal models or, the role of IL-1 downstream of TNF may not be sufficiently distinct to offer any benefit on blocking TNF.

## 1.4 Psoriatic arthritis

PsA is characterised by stiffness, pain and swelling in the joints of the hands and feet and affects approximately 23% of people with psoriasis. PsA is distinguished from RA because it presents equally in men and women. Furthermore, inflammation of the peripheral joints is more common and is more likely to affect all joints of a single digit rather than the same joints on both sides of the body, which is typical of RA [396]. Much like RA, PsA can lead to chronic joint damage, increased disability and increased mortality. Disease activity of PsA patients is measured by the Psoriatic Arthritis Response Criteria (PsARC), which includes an assessment of 78 tender joints, 76 swollen joints, a global patient assessment and a global physician assessment. Skin disease is measured independently by the Psoriasis Area and Severity Index (PASI).

### *1.4.1.1 Susceptibility to psoriatic arthritis*

Much like RA, PsA has been shown to be heritable; the risk of developing disease in first-degree relatives of individuals with PsA was 17.7%, notably more than the risk of developing psoriasis in first degree relatives, which was only 7.7% [397]. There have been few genome-wide association studies (GWAS) of PsA susceptibility genes and those that have been done often do not have the necessary power to confirm differences. Moreover, PsA studies are complicated by the co-presentation of psoriasis, for which the genetic susceptibility genes are more widely known [398]. Although there are described similarities between skin and joint inflammation in patients with PsA [399], patients with PsA always present with psoriasis, but patients with psoriasis do not always develop PsA and this disparity may be based on genetic

differences. Interestingly, like RA, SNPs associated with susceptibility to PsA include TNFR signalling pathway members such as A20 and members of the NFκB pathway [398, 400]. This suggests that common factors may be associated with the development of all rheumatic autoimmune diseases.

Whilst no GWAS studies have investigated PsA patients specifically, within the psoriasis studies there is a subset of patients with PsA. This provides an insight into genetic polymorphisms that might result in the development of PsA in patients with psoriasis. Compared to healthy controls, psoriasis patients with concomitant PsA show differences in the genes which encode the class I MHC molecule, in particular HLA-C, the IL-12 family member IL-12B and the TNFR signalling family member TNFAIP3-interacting protein 1 (TNIP1). Furthermore, studies have found an IL-1α SNP variant that is associated with the development of PsA [401]. In psoriasis, SNPs in genes coding for Th2 cytokines have been associated with disease development. Similarly, a SNP in the IL-4 receptor has been associated with joint destruction in PsA and a SNP in the IL-13 gene has been shown to be predictive of PsA development in patients with psoriasis [402, 403]. A more recent study has identified a susceptibility locus at the NFκB family member, REL [404].

Environmental factors that have been associated with the development of PsA include rubella vaccination, injury, recurrent oral ulcers, fractured bones and HIV [405, 406].

#### *1.4.1.2 Immunopathogenesis of psoriatic arthritis*

Fifty percent of patients with PsA show radiographic lesions at 1 year [407] but PsA has been found to bear more similarity to the spondyloarthropathies such as ankylosing spondylitis (AS), than RA. This is due to increased vascularity and neutrophil infiltration of the synovium in comparison to patients with RA, but similar levels to patients with AS [408]. Moreover, PsA patients can develop inflammation at the tendon or ligament attachment sites (enthesis) [409], a feature common in patients with spondyloarthropathies.

Levels of IL-22 are elevated in the synovial fluid of PsA patients and can drive the proliferation of FLS [410]. Further analysis of the synovium of PsA patients found elevated levels of TNF, IL-1β, IL-18, IL-6, IL-2, IL-10, and IFNγ [411, 412]. Furthermore, the synovium of patients with PsA has increased VEGF and angioproteins [413], large numbers of osteoclasts and alterations in the RANKL:OPG ratio [414]. T cell and plasma B cell numbers are lower in PsA patients than in patients with RA [412]. Moreover, there is less pronounced synovial hyperplasia and fewer monocytes and macrophages in patients with PsA than RA [415]. In contrast to RA, CD8 T cells and not CD4 cells are important for disease progression and activated T cells have been identified in both the skin and the joint [416].

A model has been proposed that links skin and joint inflammation in PsA. CCL22 is the ligand of CCR4, a chemokine receptor vital for homing to skin. In patients with PsA, CCL22 production has been detected in the synovium; this may allow

pathogenic skin homing psoriatic T cells to traffic to the joint and drive synovitis [417].

### 1.4.2 Therapy for PsA

Treatment of PsA is similar to RA although the therapeutic options are more limited. Methotrexate and sulfalsalazine are used first; the former is preferred as it has an additional therapeutic effect on the skin disease. Methotrexate has been shown to reduce CCL8 expression, E-selectin expression, ICAM-1 and MMP-3 in the synovium of patients with PsA. Additionally, synovial lining thickness was reduced, but vascularity was unaffected [418].

### 1.4.3 Anti-TNF therapy for the treatment of psoriatic arthritis

PsA patients too can be prescribed anti-TNF therapy, if patients have 3 or more tender joints and 3 or more swollen joints and have failed at least 2 DMARDS. It has been shown that osteoclast precursor cells are reduced in the periphery of patients with PsA treated with anti-TNF [419]. Thus, treatment with anti-TNF has the potential to reduce joint damage in PsA. Indeed, PsA patients treated with adalimumab were shown to have decreased T cells and MMP-13 expression in the synovium [420] and etanercept has been shown to decrease the progression of radiographic damage in patients with PsA and to promote joint repair [421, 422].

## 1.5 Hypotheses and Aims

During autoimmune inflammation cytokines such as TNF and IL-17 drive vascularisation of the synovium, the production of chemokines, the upregulation of adhesion molecules for further cellular recruitment, the production of matrix degrading enzymes from cells of the synovium, osteoclastogenesis, maturation of cells, the inhibition of Treg suppressive function and the promotion of Treg instability. The blockade of inflammatory cytokines and in particular anti-TNF therapy has been shown to be effective in the treatment of both RA and PsA. In this thesis I sought to investigate the mechanisms through which anti-TNF therapy mediates the amelioration of disease in patients with RA and PsA.

Patients with PsA and RA demonstrate similar clinical response to monoclonal anti-TNF antibodies or soluble TNF receptor therapy. Nevertheless, patients can fail one anti-TNF yet go on to respond to another. Moreover, in granulomatous diseases such as Chron's disease monoclonal anti-TNF antibody, but not the soluble receptor is an effective therapy. Previous studies by our group have shown that infliximab therapy in patients with RA can induce a population of Treg that suppress IFNγ via the production of IL-10 and TGFβ. Furthermore, these anti-inflammatory cytokines have been shown to suppress IL-17, a key inflammatory cytokine in arthritis and skin disorders.

**Hypotheses:**

Based on the findings described, the aim of this thesis was to test the following hypotheses:

- Anti-TNF monoclonal antibodies and anti-TNF soluble receptor therapy have different mechanisms of action – only anti-TNF monoclonal antibodies induce Treg.
- Treg induced by anti-TNF monoclonal antibodies suppress IL-17 production via the production of IL-10 and TGFβ.

Additionally, data in this thesis suggests that IL-6 regulates Th17 cells and thus a third hypothesis was formed:

- Based on the ability of IL-6 to control Th17 cells in RA patients treated with adalimumab, the therapeutic blockade of IL-6 in RA patients would result in a decrease in Th17 cells.

**Aims:**

To test these hypotheses this study will;

1. Determine if the induction of Treg is a common feature of response to all anti-TNF therapies in RA and PsA.
2. Examine the differences in immune cell subsets in RA and PsA patients treated with adalimumab and etanercept.
3. Investigate if Treg induced by anti-TNF therapy can suppress Th17 responses.
4. Explore the underlying mechanisms of Th17 cell suppression.
5. Elucidate the effects of tocilizumab therapy on Th17 cells.

# Chapter 2

# Materials and Methods

## 2.1 Sample Collection

Full consent was obtained from all participants in this study (ref: 02/0240) (Appendix I). Healthy volunteers were staff and students within the Centre for Rheumatology at the Windeyer Building, UCL. 50ml of blood was taken from patients requiring routine blood tests. All subjects were given a numerical code before storage in order to maintain anonymity. Patients classified as active were those awaiting anti-TNF therapy and had a DAS28 >5.1. Patients were considered responders if the DAS score fell by more than 1.2 and the CRP was below 5.

### 2.1.1 Preparation of Peripheral Blood Mononuclear Cells (PBMC)

Blood was collected in a 50ml universal containing 100U of preservative free heparin (CD pharmaceuticals) and processed on the same day. Blood was diluted in an equal volume of complete medium (RPMI 1640) (SIGMA-Aldrich) supplemented with 100U/ml penicillin and 100μg/ml streptomycin (SIGMA-Aldrich) and 10% heat inactivated fetal calf serum (FCS) (BIOSERA). The blood was then layered on to 12ml of Ficoll-paque (GE Healthcare) and centrifuged in a Mistral 3000i for 35minutes, 850 x g at room temperature without brake. The PBMC interface was removed and mixed with complete medium and centrifuged for 10 minutes, 500 x g at 4°C. The pellet was resuspended in 10ml complete medium and counted as

described in 2.1.2. Aliquots of cells were removed as required for *ex vivo* staining, before the cells were centrifuged and frozen as described in section 2.1.3.

### 2.1.2 Counting Cells

10µl of cell suspension were mixed with 90µl of 0.4% trypan blue (SIGMA-Aldrich). The suspension was transferred to a Neubauer counting chamber and cells were counted using light microscopy. Cells that were bright were counted, and cells that appeared blue were excluded. Each of the 4 corner squares were counted, 1 corner square contained 16 squares and the cell number within this 4 x 4 chamber represented $10^4$ cells. Thus, the number of cells was calculated as follows:

$$\frac{\text{Total number of cells counted}}{4} \; x \text{ dilution factor } x \; 10^4 = \text{number of cells/ml}$$

### 2.1.3 Freezing cells

Once PBMC had been isolated from blood, they were resuspended at a concentration of $2x10^7$ cells/ml in freezing medium (see below). One to two ml aliquots were transferred into cryovials (NUNC) and stored in a freezing box containing isopropanol at –70°C for 1-2 days before transfer to liquid nitrogen storage.

**Freezing medium:** Heat inactivated FCS supplemented with 10 % v/v dimethyl sulphoxide (SIGMA-Aldrich).

### 2.1.4 Thawing Cells

Cells stored in liquid nitrogen were thawed rapidly in a 37°C water bath (Grant), transferred to a universal tube and complete medium was added in a drop-wise fashion. The tube was centrifuged at 400 x g for 5 minutes at 4°C, and washed once more before counting (Section 2.1.2).

## 2.2 Fluorescent Activated Cell Sorting (FACS)

### 2.2.1 Buffers and Solutions

FACS buffer: 1 x phosphate buffered saline (PBS), 1% FCS, 0.1% sodium azide

MACS buffer: 1 x PBS (SIGMA-Aldrich), 1% FCS,

2mM Ethylenediaminetetraacetic acid (EDTA)

### 2.2.2 Cell Surface and Intracellular Staining

Cells were centrifuged at 4°C, 500 x g for 5 minutes and washed in FACS buffer, before vortexing briefly and adding 20µl of appropriate surface antibodies at the titrated dilution (Table 2.1, pg 108) for 20 minutes at 4°C. Cells were washed twice in 100µl of FACS buffer, before adding fixation/permeabilisation buffer (eBioscience) for 30 minutes at 4°C. Cells were centrifuged and washed twice in permeabilisation buffer (eBioscience) before the addition of intracellular antibodies at the appropriate concentration (Table 2.1, pg 108) for 1 hour at 4°C. When staining for transcription factors, after washing with permeabilisation buffer, cells were incubated for 10 minutes with 20µl 0.1µg/ml human immunoglobulin (SIGMA-

Aldrich) per $10^6$ cells, followed by the addition of intracellular antibodies for 45 minutes. Cells were then washed twice in 100µl permeabilisation buffer before being resuspended in FACS buffer to be acquired within 48 hours on the BD FACS Calibur, BD LSR, BD LSRII or the BD Fortessa.

**Table 2.1 Antibodies used for flow cytometry with human samples**

| Antibody | Fluorochrome | Clone | Isotype | Dilution | Company |
|---|---|---|---|---|---|
| CD4 | FITC | RPA-T4 | Mouse IgG1 κ | 1:20 | BD Pharmingen |
| | PE | OKT4 | Mouse IgG2b | 1:5 | eBioscience |
| | PE-Cy7 | RPA-T4 | Mouse IgG1 κ | 1:50 | eBioscience |
| | Alexa Fluor 700 | RPA-T4 | Mouse IgG1 κ | 1:40 | BD Pharmingen |
| CD25 | PE | M-A251 | Mouse IgG1 κ | 1:10 | BD Pharmingen |
| | PE-Cy7 | 2A3 | Mouse IgG1 κ | 1:5 | BD Pharmingen |
| | APC-Cy7 | M-A251 | Mouse IgG1 κ | 1:5 | BD Pharmingen |
| CD127 | PE-Cy5 | ebioRDR5 | Mouse IgG1 | 1:40 | eBioscience |
| FOXP3 | Alexa Fluor 647 | PCH101 | Rat IgG2a | 1:40 | eBioscience |
| | PE | 236AIE7 | Mouse IgG1 | 1:40 | eBioscience |
| RORγt | PE | AFKJS-9 | Rat IgG2a | 1:200 | eBioscience |
| IFNγ | PE-Cy7 | B27 | Mouse IgG1 κ | 1:60 | BD Pharmingen |
| IL-17A | Alexa Fluor 647 | BL168 | Mouse IgG1 | 1:50 | Biolegend |
| IL-22 | APC | 142928 | Mouse IgG1 | 1:10 | R&D Systems |
| CD14 | PE | 61D3 | Mouse IgG1 | 1:50 | eBioscience |
| CD24 | PE | EBioSN3 | Mouse IgG1 | 1:20 | eBioscience |
| TGF-βRII | PE | | Goat IgG | 1:5 | R&D |

| | | | | | |
|---|---|---|---|---|---|
| CD39 | PE | EBioA1 | Mouse IgG1 | 1:20 | eBioscience |
| STAT3 (pS727) | Alexa Fluor 647 | 49/p-stat3 | Mouse IgG1 | 1:5 | BD Pharmingen |
| STAT3 (pY705) | Alexa Fluor 647 | 4/p-STAT3 | Mouse IgG2a κ | 1:5 | BD Pharmingen |
| CD62L | PE-Cy5 | DREG-56 | Mouse IgG1 κ | 1:10 | BD Pharmingen |
| TNFα | FITC | MAbII | Mouse IgG1 k | 1:20 | eBioscience |
| Ki67 | FITC | B56 | Mouse IgG1 κ | 1::40 | BD Pharmingen |
| CD27 | APC H7 | M-T271 | Mouse IgG1 κ | 1:40 | BD Pharmingen |
| | PE | M-T271 | Mouse IgG1 κ | 1:40 | BD Pharmingen |
| α ms/hu Helios | PE | 22F6 | Armenian Hamster IgG | 1:20 | Biolegend |
| CD19 | APC | HIB19 | Mouse IgG1 κ | 1:40 | eBioscience |
| | PE-CY7 | HIB19 | Mouse IgG1 κ | 1:40 | eBioscience |
| | V450 | HIB19 | Mouse IgG1 κ | 1:20 | BD Pharmingen |
| CD38 | PE-Cy7 | HIT2 | Mouse IgG1 κ | 1:40 | eBioscience |
| IL-10 | Alexa-Fluor 647 | JES3 | Rat IgG1 κ | 1:20 | eBioscience |
| TGF-β | PE | 9016 | Mouse IgG1 | 1:20 | R&D |
| CD73 | FITC | AD2 | Mouse IgG1 κ | 1:10 | eBioscience |
| IgD | FITC | IA6-2 | Mouse IgG2a κ | 1:5 | BD Pharmingen |
| IL-21 | Alexa Fluor 647 | EBio3A3-N2 | Mouse IgG1 κ | 1:20 | eBioscience |

Unless otherwise specified, all antibodies were generated against human proteins.

### 2.2.3 Staining for apoptotic cell markers

Cells were stained with Annexin V PE and 7AAD (7-Aminoactinomycin D) using the PE Annexin V Apoptosis Detection Kit I (BD). Cells were washed in FACS buffer and surface stained as described in section 2.2.2. Samples were then washed in 1 x Annexin V binding buffer before the addition of Annexin V PE (1:40 dilution) and 7AAD (1:40 dilution) for 15 minutes at room temperature. Samples were resuspended in 150μl of Annexin buffer and the data were acquired on the BD Calibur within 1 hour.

### 2.2.4 Staining cells for FOXP3 and pSTAT3

Five hundred thousand PBMC were plated in a 96 well u-bottomed plate at a final volume of 200μl. Cells were left unstimulated or stimulated with 100ng/ml recombinant human IL-6 (Peprotech) or 1μg/ml soluble anti-CD3 and 1μg/ml anti-CD28 antibodies (eBioscience). After incubation the plate was centrifuged briefly (30 seconds, 800 x g, 4°C) before the addition of BD Phosflow fix buffer 1 (BD) which had been warmed to 37°C. Cells were incubated in fix buffer for 10-15 minutes at room temperature in the dark before washing in FACS buffer. Cells were then stained with CD4 PE-Cy7 at a concentration optimised for these buffers (1:20) for 20 minutes, washed in 100μl of FACS buffer and resuspended in 1 x Phosflow Perm/Wash buffer I (BD) for a further 15 minutes. Antibodies against STAT proteins were added in a 1:5 dilution and FOXP3 PE antibodies were added at a concentration optimised for the Phosflow buffers (1:15) for 30-45 minutes at 4°C. Cells were

washed once in buffer I and resuspended in FACS buffer for acquisition on the BD FACS Calibur.

When responder T cells and Treg isolated by FACS (using markers described in section 2.3.2) were stained for pSTAT3, cells were rested for 1 hour (37°C, 5% $CO_2$) and then fixed with BD Phosflow fix buffer 1 (BD) as above. As these were isolated populations and the purity was checked separately, there was no need to stain cells for surface markers or FOXP3. So, cells were incubated with Phosflow Perm buffer III for 15 minutes at 4°C before the addition of STAT3 antibodies for 30-45 minutes at the same temperature. Cells were washed in buffer III and resuspended in FACS buffer for acquisition on the BD FACS Calibur within 48 hours.

The fold induction of pSTAT3 was calculated as follows:
MFI = mean fluorescence intensity

$$\frac{\text{pSTAT3 MFI stimulated - pSTAT3 MFI unstimulated}}{\text{pSTAT3 MFI unstimulated}}$$

### 2.2.5 Preparation of compensation beads

When the BD LSRII was used for large flow cytometry staining panels, a human Comp Beads Set (BD) (Mouse IgG κ) was used to prepare compensation controls for the machine. One hundred µl of FACS buffer was added to a FACS tube (BD). Bottles containing positive and negative sets of beads were vortexed for 15 seconds before the addition of 1 drop from each bottle to the FACS buffer. One FACS tube

was an unstained control and to the others 2μl of the desired antibody were added before incubating at room temperature for 15-30 minutes. One ml of FACS buffer was added to each tube, they were centrifuged at 800 x g for 30 seconds and resuspended in 150μl FACS buffer.

### 2.2.6 Flow cytometry data analysis

After data acquisition on CellQuest (BD), CellQuest Pro (BD) or FACSDiva (BD) software, data were analysed using FlowJo version 8.8.4 or version 9.3.3 (Tree Star Inc.). Live cells were gated based on forward and side scatter properties (Figure 2.1 A).

### 2.2.7 Preparing cells for sorting

Cells were thawed in complete medium before being washed twice (10 minutes, 450 x g, 4°C) in 50ml of MACS buffer. The pellet was stained using antibodies against CD4, CD25 and CD127 (1μl/$10^6$ cells), as this cell population is known to contain the majority of the $FOXP3^+$ Treg fraction (Figure 2.1B). Antibodies were incubated for 20 minutes at room temperature in the dark before washing and resuspending in approximately 1ml of MACS buffer. Cell suspensions were then passed through 35μM cell strainer cap FACS tubes (BD) and kept on ice. Cells were sorted using the BD FACS Aria and collected in polypropylene FACS tubes (BD) containing media comprising 50% RPMI 1640 and 50% FCS.

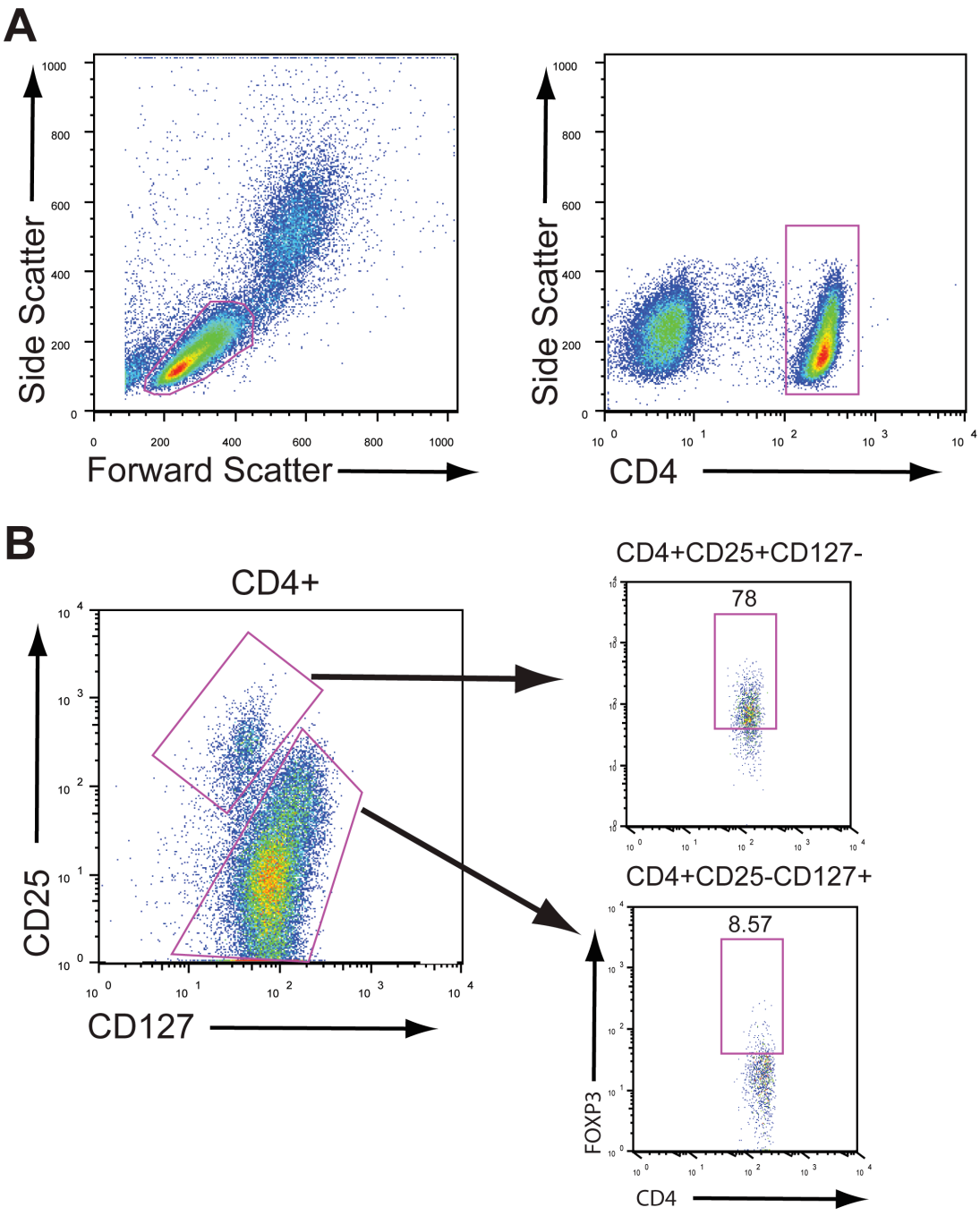


**Figure 2.1. Gating strategies used for flow cytometry**
**A.** Lymphocytes were distinguished from larger cells by forward and side scatter properties. From this the $CD4^+$ population was identified by high expression of this marker. **B.** CD25 and CD127 expression was examined on the $CD4^+$ population. Subsequently the percentage of $FOXP3^+$ cells in the $CD4^+CD25^+CD127^-$ population was compared to the $CD4^+CD25^-CD127^+$ population.

## 2.3 Functional regulatory T cell assays

### 2.3.1 Treg Depletion

Cells were prepared for FACS sorting (described in section 2.2.7) and each sample was divided into two tubes. For each sample one tube was passed through the FACS Aria and depleted of Treg, the second tube was passed through the machine but no cells were depleted. Samples were pooled and washed in complete medium (450 x g, 10 minutes, 4°C) before being counted as described in 2.1.2. Cells were rested at 37°C for 1 hour before being centrifuged at 450 x g, 21°C for 10 minutes. The pellet of each sample was resuspended in complete medium and $3x10^5$ cells were added to each well in a 96 well u-bottomed plate with a final volume of 200µl. The well was supplemented with 1µg/ml anti-CD3 (HIT3a eBioscience) and 1µg/ml anti-CD28 (CD28.2 eBioscience) and incubated at 37°C in 5% $CO_2$ for 3 days. Remaining cells were stained to determine the purity of the sort, which was typically over 92%. Additionally cells were stained for FOXP3 to determine the purity of the Treg fraction (Figure 2.2A). On day 3, 150µl of supernatant were removed for ELISA and supplemented with 150µl of stimulation mix, consisting of PMA (4 µg/ml) (1:100, ionomycin (1 µg/ml) and Golgi Stop™ (BD) (2µl/ml), and returned to the incubator for 4 hours. The cells were then stained for IL-17 Alexa Fluor 647 and IL-22 APC using the antibody dilutions outlined in table 2.1 (pg 108) and the staining protocol described in section 2.2.2. The data were acquired on the BD LSR.

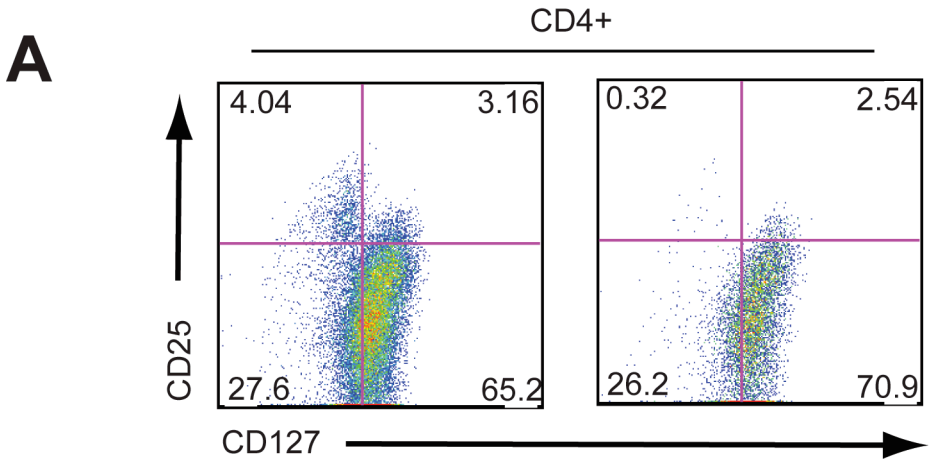


**Figure 2.2. Purity of FACS sorted cell populations.**
**A.** Cells were prepared for sort by staining with CD4, CD25 and CD127 and subsequently stained with FOXP3. The left panel shows a distinctive population of $CD4^{+}CD25^{+}CD127^{-}$ cells, which has been depleted in the right panel.

### 2.3.2 Tresponder-Treg co-culture

FACS sorting (Section 2.2.7) was used to isolate two cell populations: $CD4^{+}CD25^{+}CD127^{-}$ (Treg) cells and $CD4^{+}CD25^{-}CD127^{+}$ (responder T) cells. Samples were centrifuged (10 minutes 450 x g, 21°C) before being resuspended in complete media for counting (Section 2.1.2). After resting for 1 hour in a 37°C incubator, cells were centrifuged (10 minutes 450 x g, 21°C) and resuspended in complete media at a concentration of $1x10^{5}$ cells/100µl. Four conditions were set up for each experiment:

1. Unstimulated Tresp

2. Stimulated Tresp

3. Stimulated Treg

4. 3:1 ratio of Tresp to Treg (stimulated).

Cells were stimulated with 1μg/ml soluble anti-CD3 and 1μg/ml anti-CD28 antibodies in the appropriate wells. Remaining cells were stained to determine the purity of the sort, typically above 92% (Figure 2.3).

After 3 days, 150μl of supernatant were transferred to a 96 well flat-bottomed plate and stored at –20°C for ELISA. Cells were supplemented with 150μl of stimulation mix, made as above. The plate was returned to the incubator for an additional 4 hours. The cells were then stained for IL-17 Alexa Fluor 647 and IFNγ PE-Cy7 using the antibody dilutions in table 2.1 (pg. 108) and the staining protocol described in section 2.2.2. Data were acquired on the BD LSR.

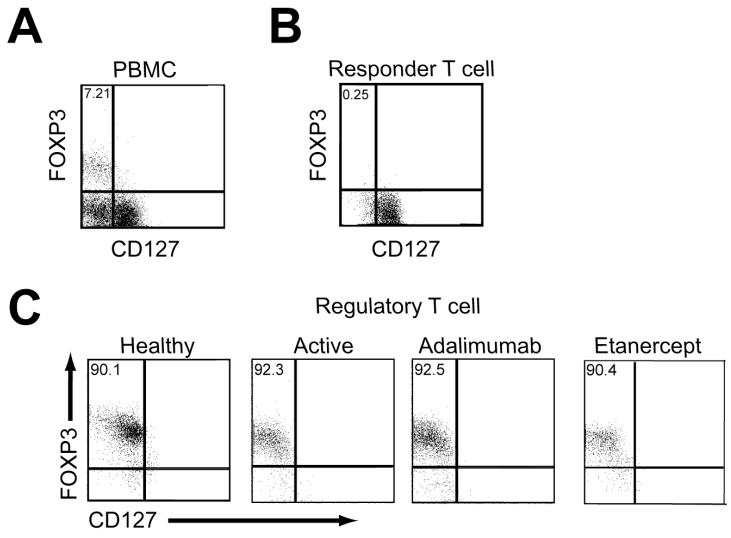


**Figure 2.3. The purity of Treg after FACS sorting in healthy controls, patients with active RA and patients treated with adalimumab or etanercept.**
Responder T cells and Treg were isolated by FACS sorting from PBMC. Representative FACS plots show FOXP3 staining of **A.** whole PBMC, **B.** isolated responder T cells and **C.** regulatory T cells from healthy controls, patients with active RA, patients treated with adalimumab and patients treated with etanercept.

### 2.3.3 Tresponder-Treg-monocyte co-culture

FACS sorting (Section 2.2.7) was used to isolate Treg and Tresp as described in 2.3.2 and monocytes were isolated based on expression of CD14. Monocytes were stained with PKH-26 cell membrane dye using the protocol outlined in section 2.3.9. Samples were centrifuged before being counted (Section 2.1.2). Tresp were resuspended in complete media at $2x10^6$ cells/ml and Treg and monocytes were each resuspended at $6.6x10^5$ cells/ml. Cells were set up in a final volume of 200µl in a ratio of 3 Tresp:1 monocyte or 3 Tresp:1 monocyte:1 Treg. All conditions were stimulated with 1µg/ml soluble anti-CD3 and 1µg/ml anti-CD28 antibodies for 5 days at 37°C and 5% $CO_2$.

After 5 days 150µl of supernatant were transferred to a 96 well flat-bottomed plate and stored at –20°C for ELISA. Cells were supplemented with 150µl of stimulation mix, made as above. The plate was returned to the incubator for an additional 4 hours. The cells were then stained for IL-17 Alexa Fluor 647 and IFNγ PE-Cy7 using the antibody dilutions in table 2.1 (pg. 108) and the staining protocol described in section 2.2.2. Data were acquired on the BD LSR II.

As required, additional conditions were used to investigate mechanism of suppression of Treg from adalimumab treated patients. These are described below;

### 2.3.4 Cytokine and cytokine receptor blockade

Cells were sorted as described in section 2.3.3 and were plated at the same concentration in the following conditions:

Tresp + monocytes

Tresp + monocytes + isotype control

Tresp + monocytes + αIL-6/αIL-1

Tresp + monocytes + Treg

Tresp + monocytes + Treg + isotype control

Tresp + monocytes + Treg + αTGF-β/αIL-10/αIL-10Rα/αp35

Neutralising antibodies were used at the concentrations shown in table 2.2 (pg. 118).

In some conditions 20ng/ml recombinant human IL-6 (Peprotech) was added back to cultures of Tresp, monocytes and Treg from adalimumab treated patients.

**Table 2.2 Neutralising antibodies used in Treg suppression assays**

| Antibody | Clone | Isotype | Concentration | Company |
|---|---|---|---|---|
| anti-TGF-β | 27235 | Mouse IgG1 | 10μg/ml | R&D |
| anti-IL-10 | | Goat IgG | 10μg/ml | R&D |
| anti-IL-10Rα | | Goat IgG | 10μg/ml | R&D |
| anti-IL-6 | | Goat IgG | 0.1 μg/ml | R&D |
| anti-IL-1β | | Goat IgG | 0.1 μg/ml | R&D |
| anti-p35 | 27537 | Mouse IgG1 | 10μg/ml | R&D |

### 2.3.5 Blocking CD39 activity

The working concentration of the anti-CD39 antibody (Table 2.3, pg 119) was determined to be 2μg/ml in experiments detailed in section 2.3.6.

**Table 2.3 Anti-CD39 neutralising antibody clone and working concentration**

| Antibody | Clone | Isotype | Concentration | Company |
|---|---|---|---|---|
| anti-CD39 | A1 | Mouse IgG1κ | 2μg/ml | Biolegend |

Thus, upon isolation, each population of cells was divided in two. One half of each isolated cell subset was untreated and the other half was treated with anti-CD39 antibody. Both were placed in a 37°C water bath for 30 minutes before resuspending cells at the concentration described in section 2.3.3. Cells were then plated a 96 well u-bottomed plate in the following conditions and stimulated as described above (section 2.3.3):

Tresp + monocytes

Tresp + monocytes + Treg

Tresp + monocytes + anti-CD39 treated Treg

anti-CD39 treated Tresp + monocytes

Tresp + anti-CD39 treated monocytes

Cells were stimulated and cultured for 5 days as described in section 2.3.3, then re-stimulated and stained as described in section 2.2.2.

### 2.3.6 ATP consumption Assay

Whole PBMC were thawed, counted and separated into 2 populations. Two μg/ml anti-CD39 was added to one population and the equivalent volume of complete medium was added to the other. Cells were incubated at 37°C for 30 minutes, washed and counted again. Each population was plated at 2.5 x $10^5$ cells/well with at least 2 replicates.

The ATP consumption assay was performed by Halima Moncrieffe, of the Immunopathogenesis Group at UCL Institute of Child Health, using a protocol described in a previous study [101]. The method from that paper is included below:

*"A total of 2.5 x $10^4$ cells/well were incubated at room temperature for 10 minutes in the presence of 25 μM ATP (Sigma-Aldrich). CellTiter-Glo reagent from the Luminescent Cell Viability Assay (Promega, Madison, WI) was added as per manufacturer's instructions. CellTiter-Glo contains a source of luciferase, which, in the presence of ATP, releases luminescence that was rapidly measured using an Optima luminometer (BMG Labtech, Aylesbury, U.K.). ATPase activity was measured by a reduction in luminescence (light units) during the assay."*

Dr Moncrieffe collected the data and generated the graph shown in figure 4.7B.

### 2.3.7 STAT3 inhibitor

PBMC were sorted as described in section 2.3.3. Tresp, monocytes and Treg were plated in separate wells of a 96 well u-bottomed plate at the concentrations described

in section 2.3.3. Isolated cell subsets were placed in a 37°C, 5% $CO_2$ incubator overnight in complete medium or in STAT3 inhibitor VII (Calbiochem) at 5μg/ml in 0.02% DMSO. The following morning cells were combined in the following conditions:

Tresp + monocytes

Tresp treated with inhibitor + monocytes

Tresp + monocytes + Treg treated with inhibitor

Tresp + monocytes + Treg

Cells were stimulated and cultured for 5 days as described in section 2.3.3, then re-stimulated and stained as described in section 2.2.2.

### 2.3.8 Transwell Experiments

A 96 well HTS Transwell permeable support with a 0.4μm pore size polycarbonate membrane was purchased from Corning Inc. Three hours before plating FACS sorted cells, the transwell plate was equilibrated by the addition of 175μl of complete medium to the bottom chamber. The upper chambers were carefully overlaid and a further 50μl medium to each of these compartments and the plate was placed in a 37°C, 5% $CO_2$ incubator. Tresp were resuspended at $4x10^6$ cells/ml and Treg and monocytes were resuspended at $1.32x10^6$ cells/ml so that cells could be added in a volume of 25μl in the following conditions:

Tresp + monocytes (lower chamber)

Tresp + monocytes + Treg (lower chamber)

Tresp + monocytes (lower chamber) + Treg (upper chamber)

One μg/ml of anti-CD3/28 was added in a volume of 10μl to the lower chamber. The final volume in each well was 235μl. Cells were cultured for 5 days.

On day 5, the medium was removed from the upper and lower chamber of the transwell, transferred to a 96-well u-bottom plate and centrifuged for 5 minutes at 350 x g 21°C. The supernatant was removed, transferred a flat-bottomed 96 well plate and stored at –20°C. Fifty μl of cell dissociation buffer (Gibco) was added to both the upper and lower chambers of the transwell plate and mixed by pipette then transferred to the 96 well u-bottom plate containing the cells and centrifuged as above. Cells were supplemented with 200μl of stimulation mix for 4 hours in a 37°C, 5% $CO_2$ incubator and stained as described in section 2.2.2.

### 2.3.9 PKH-26 Staining of monocytes

PKH-26 dye (SIGMA-Aldrich) was diluted 1:1000 in diluent C (SIGMA-Aldrich). One ml of the diluted dye was added per $10^7$ cells for 3 minutes in the dark. An equal volume of room temperature FCS was added for a further 3 minutes to stop the reaction. Cells were washed twice in complete medium before counting and resuspending in the correct volume.

### 2.3.10 Calculating Suppression

*CP* = cytokine production.

Suppressive ability of Treg in depletion experiments was calculated as follows:

$$\frac{\text{Treg depleted PBMC } CP - \text{Whole PBMC } CP}{\text{Whole PBMC } CP} \; x\ 100$$

Suppression in the co-culture experiments was calculated as follows:

$$\frac{\text{Tresp } CP - (\text{Tresp} + \text{Treg})\ CP}{\text{Tresp } CP} \; x\ 100$$

or

$$\frac{(\text{Tresp} + \text{monocytes})\ CP - (\text{Tresp} + \text{monocytes} + \text{Treg})\ CP}{(\text{Tresp} + \text{monocytes})\ CP} \; x\ 100$$

## 2.4 Mouse immunisation and flow cytometry staining

C57BL/6 mice (Charles River) were maintained in specific pathogen-free facilities under home office guidelines. Mice were immunised with methylated Bovine Serum Albumin (mBSA - SIGMA-Aldrich) dissolved in water to a concentration of 40 mg/ml. One hundred μg of mBSA were mixed 1:1 with Complete Freund's adjuvant and 100μl was injected at the base of the tail. Fourteen days later, mice were boosted with 100μg mBSA mixed 1:1 in incomplete Freund's adjuvant in the base of the tail.

Three days later mice were sacrificed under Schedule 1. All animal handling components were undertaken by Dr. Clare Notley. The inguinal draining lymph nodes were removed and a single cell suspension prepared by passing the lymph node through a 0.7μm cell strainer. Cells were washed in complete medium (prepared as previously described, and additionally supplemented with 50μM 2-Mercaptoethanol- SIGMA-Aldrich) and plated at $5x10^5$ cells/well in a 96 well u-bottom plate. Cells were stimulated with 1ng/ml PMA, 1μg/ml ionomycin and 1μl/ml Golgi Stop for 4 hours at 37°C. After this time the cells were stained for CD4 FITC (RM4-5, 1:200, dilution BD Pharmingen), IL-17A Alexa Fluor 647 (eBio17B7, 1:50, eBioscience) and RORγt (see table 2.1, pg 108). The protocol used was the same as described in section 2.2.2. Data were acquired on the BD FACS Calibur.

## 2.5 Enzyme-linked Immunosorbent Assay (ELISA)

### 2.5.1 Buffers and reagents

1xTBS: 20ml 1 x Tris Buffer (pH 7.2), 8.7g NaCl

Blocking buffer: 1 x PBS (SIGMA-Aldrich), 1% BSA

Dilution Buffer: 1 x TBS, 1% BSA, 0.05% Tween 20

Wash Buffer: 1 x PBS, 0.05% Tween 20

### 2.5.2 IL-17 ELISA

Ninety-six well NUNC maxisorb (NUNC) plates were coated overnight at room temperature with 50µl of 4µg/ml anti-human IL-17 antibody (R&D Systems) in dilution buffer. The plates were washed 5 times with wash buffer before incubation with 250µl of blocking buffer for 1.5 hours at room temperature. Samples stored from previous experiments were thawed and diluted as required and standard curve prepared from recombinant human IL-17 antibody (R&D Systems) with a top concentration of 2000pg/ml. The plate was washed a further 5 times, before standard curve and the samples were added to the plate and incubated for 2 hours at room temperature. After washing 5 times, 50µl of 0.5µg/ml anti-IL-17 detection antibody in dilution buffer (R&D systems) were added to the plate for 2 hours at room temperature. The plate was washed 5 times and 50µl streptavidin-horseradish peroxidase (R&D Systems) diluted 1/200 were added to the plate for 30 minutes at room temperature, before a further wash step. One hundred µl of tetramethylbenzidine (TMB) (SIGMA-Aldrich) were added to each well and allowed to develop for 5-10 minutes, the reaction was stopped by the addition of 50µl of 2M sulphuric acid. The OD values were acquired at 450nm on the MRX TC revelation (DYNEX technologies) within 1 hour.

### 2.5.3 IL-10 ELISA

The IL-10 ELISA was undertaken using the protocol described above. The reagents specific to this experiment were:

Capture antibody: anti-IL-10 Clone: Mouse IgG1 (R&D)

Detection antibody: anti-IL-10 Clone: Goat IgG (R&D)

Standard: recombinant human IL-10 (R&D)

### 2.5.4 IL-23 ELISA

IL-23 ELISA was performed with an ELISA Ready-SET-Go Set (eBioscience) and a 96 well NUNC maxisorb plate. Most of the required reagents were included, but wash buffer and stop solution (2M sulphuric acid) were prepared as above. Plates were incubated overnight with 100μl of capture antibody (anti-p19) in coating buffer (supplied). Plates were washed 5 times and blocked for 1 hour at room temperature with assay diluent (supplied). The plate was washed a further 5 times before plating serial dilutions of the standard with a top concentration of 2000 pg/ml followed by the samples at room temperature for 2 hours. The plate was washed 5 times before the addition of the capture antibody (anti-p40) for 1 hour at room temperature. The plate was washed as previously and 100μl of Avidin-HRP diluted in assay diluent as per the protocol were added to each well for 30 minutes at room temperature. The plate was washed 7 times with 1-2 minutes soaking of the wells in wash buffer between each wash. One hundred μl/well of substrate solution (provided) were added to each well for 15 minutes before the addition of 50μl stop solution to each well.

The OD values were acquired at 450nm on MRX TC revelation (DYNEX technologies).

## 2.6 Cytometric Bead Array (BD)

CBA was undertaken with a series of flex sets (BD); each cytokine of interest was associated with a different capture bead (IL-17 B5, IL-1 B4 and IL-6 A7) and reagents were prepared using a human soluble protein master buffer kit (BD). Samples were thawed and incubated with capture beads for the required cytokines in a 96-well plate for 1 hour at room temperature. PE detection reagent was added to each sample and incubated at room temperature in the dark for a further 2 hours. The plate was then centrifuged at 200 x g for 5minutes and beads were resuspended in wash buffer and centrifuged again. Each well was resuspended in 300 μl and acquired on the same day on the BD FACS Array. Samples were analysed using the BD FACS CAP program.

## 2.7 Statistical Analysis

Data were analysed using Graphpad Prism. Comparison between healthy controls and patient groups was measured using an unpaired t test. When patients were studied ‘pre’ and ‘post’ therapy, a paired t test was used. The probability value is shown on graphs as appropriate.

# Chapter 3

## Immunologic changes in rheumatoid arthritis patients treated with anti-TNF

Objectives:

1. Utilise flow cytometry to determine levels of regulatory T cells in healthy controls, patients with active RA and RA patients responding to either adalimumab or etanercept.
2. Examine Treg phenotype in healthy controls and RA patient groups using flow cytometry.
3. Determine if there are differences in key tolerogenic features in patients with active RA and RA patients treated with adalimumab or etanercept – including the *ex vivo* levels of apoptosis and production of anti-inflammatory cytokines.
4. Determine if there are differences in key pathogenic features in patients with active RA and RA patients treated with adalimumab or etanercept – including the *ex vivo* levels of $RORC^+$ Th17 cells and production of IL-17 and IFNγ.

## 3.1 RA patients responding to adalimumab, but not etanercept, have increased peripheral Treg

Previous work by this group has shown that regulatory T cells (Treg) from patients with RA are compromised in their ability to suppress cytokine production from responder T cells, in contrast to Treg from healthy controls. In RA patients treated with infliximab, it was observed that peripheral blood Treg were increased in number and were capable of suppressing IFNγ *in vitro* [124]. To confirm that the increase in Treg observed in infliximab treated patients also occurred following therapy with adalimumab, a purely human anti-TNF antibody, peripheral blood mononuclear cells (PBMC) from healthy controls, patients with active RA and RA patients responding to adalimumab were stained with flow cytometry antibodies for CD4, CD25 and FOXP3 (Figure 3.1A). As previously published [86, 124], the percentage of $CD4^+CD25^+FOXP3^+$Treg in patients with active RA was similar to healthy controls. In contrast, patients responding to adalimumab had increased peripheral Treg ($p=0.0002$). This difference was also observed if Treg were defined as $CD4^+FOXP3^+$ (Figure 3.1B). Furthermore, this increased percentage of Treg was absent in patients with persistently active disease despite treatment with adalimumab (adalimumab NR).

To determine whether this increase in Treg after therapy was explained by the neutralisation of systemic TNF, we studied a third anti-TNF therapy proven to be effective in the treatment of RA, the TNF type II receptor IgG1 fusion protein known as etanercept. The percentage of Treg in RA patients responding to etanercept was compared to healthy controls, patients with active RA and RA patients responding to adalimumab. Unexpectedly, there was no difference between the numbers of Treg (either $CD4^+CD25^+FOXP3^+$ or $CD4^+FOXP3^+$) in patients with active disease and

those responding to etanercept. In addition, the percentage of Treg was significantly lower in etanercept treated patients than in those treated with adalimumab (p=0.0007)(Figure3.1A,B).

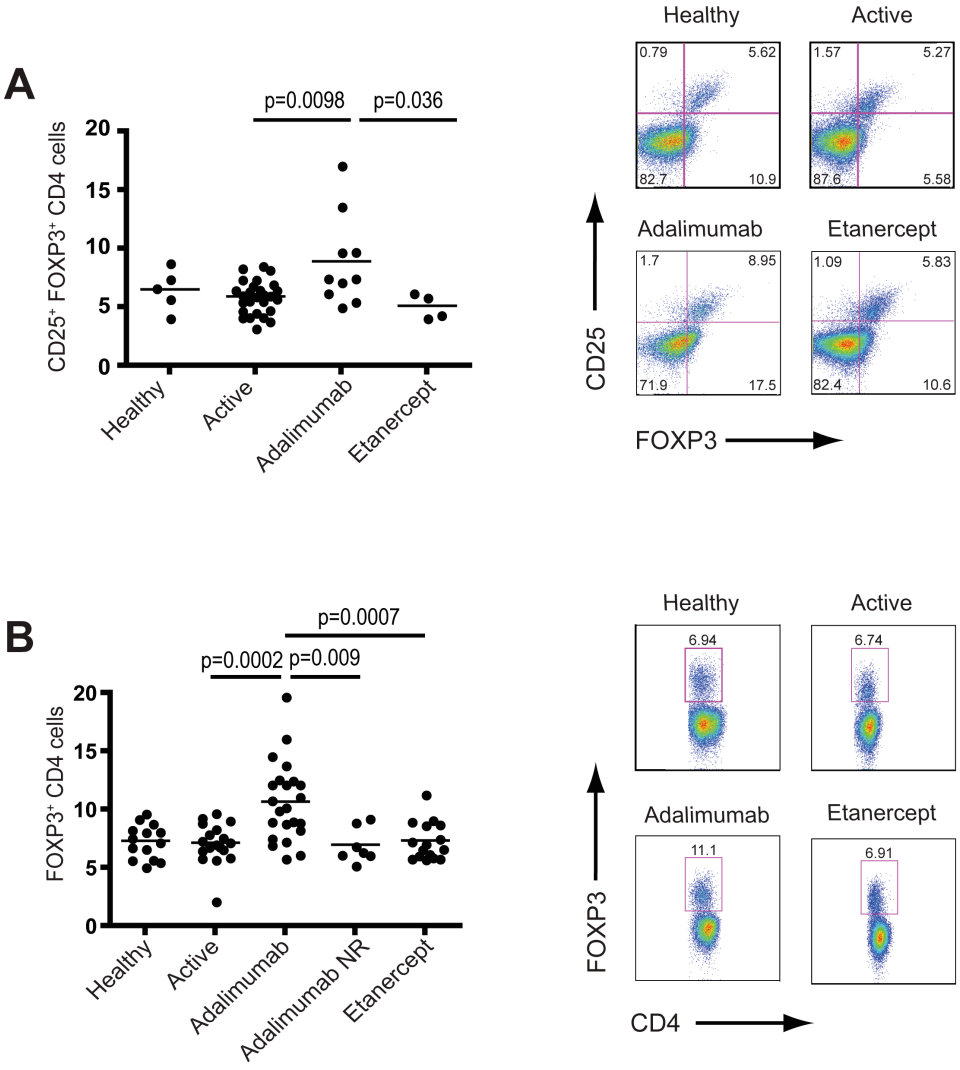


**Figure 3.1. Rheumatoid arthritis patients treated with adalimumab, but not etanercept, have increased regulatory T cells.**

*Ex vivo* PBMC from healthy controls, patients with active RA and patients responding to adalimumab or etanercept were surface stained for CD4, CD25 and the intracellular transcription factor FOXP3. **A.** Percentage of $CD4^+$ cells that are $CD25^+FOXP3^+$. Points show individual donors and bars show mean percentage $CD4^+FOXP3^+$ cells. Representative FACS plots are gated on CD4. **B.** Percentage $CD4^+FOXP3^+$ in PBMC from healthy controls, patients with active RA , patients responding to adalimumab or etanercept and patients not responding to adalimumab (adalimumab NR). Points show individual donors and bars show mean percentage $CD4^+FOXP3^+$ cells. Representative FACS plots are shown.

These differences did not appear to be dependent upon the production of autoantibodies, as the same overall trend was observed whether the patients were positive or negative for RF (Figure 3.2 A) or ACPA (Figure 3.2B).

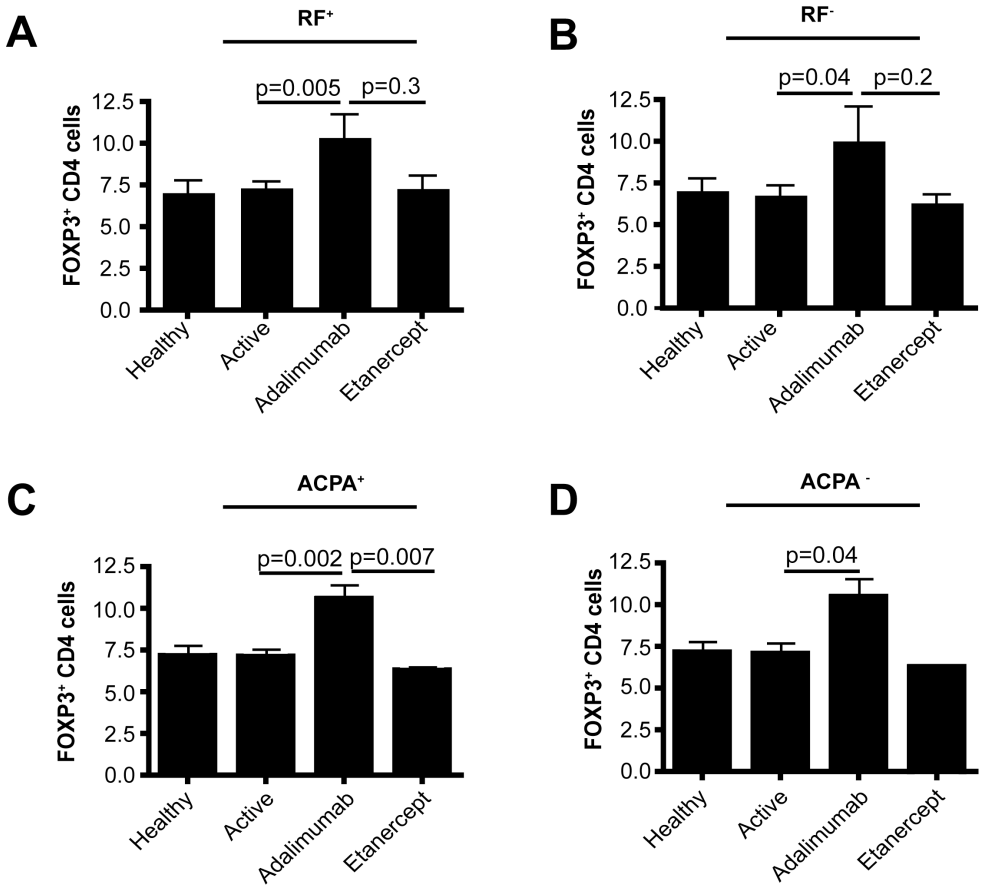


**Figure 3.2. Increased Treg after treatment with adalimumab is independent of autoantibody positivity.**

*Ex vivo* percentage of $CD4^+FOXP3^+$ cells was determined in healthy controls (*n=6*), **A.** patients positive for rheumatoid factor (active *n=20*, adalimumab *n=10*, Etanercept *n= 6*) or **B.** patients negative for rheumatoid factor (active *n=13*, adalimumab *n=5*, etanercept *n=4*), **C.** patients positive for ACPA (active *n=14*, adalimumab *n=7*, etanercept *n=4*) or **D.** patients negative for ACPA (active *n=12*, adalimumab *n=4*, etanercept *n=1*). Bars represent the mean±SE.

## 3.2 Treg from patients treated with adalimumab, but not etanercept bear the hallmarks of induced Treg.

To address whether the increased Treg observed in adalimumab treated patients represent a population of peripherally induced cells, Treg were stained for CD62L and Helios. The former has been previously described by this group as a marker of anti-TNF induced Treg [86], whilst Helios expression is thought to be confined to thymically derived Treg [72]. As expected, there was a significantly higher proportion of CD62L negative Treg in patients treated with adalimumab compared to both patients with active RA and those treated with etanercept (Figure 3.3 A). There were also more Treg from adalimumab treated patients that lacked expression of Helios compared to patients with active RA. In patients who did not respond to adalimumab, the percentage of Helios negative Treg were similar to patients with active RA (Figure 3.3 B). Notably, there were more Treg negative for both Helios and CD62L (Figure 3.3 C) in adalimumab treated patients compared to patients with active RA or patients responding to etanercept.

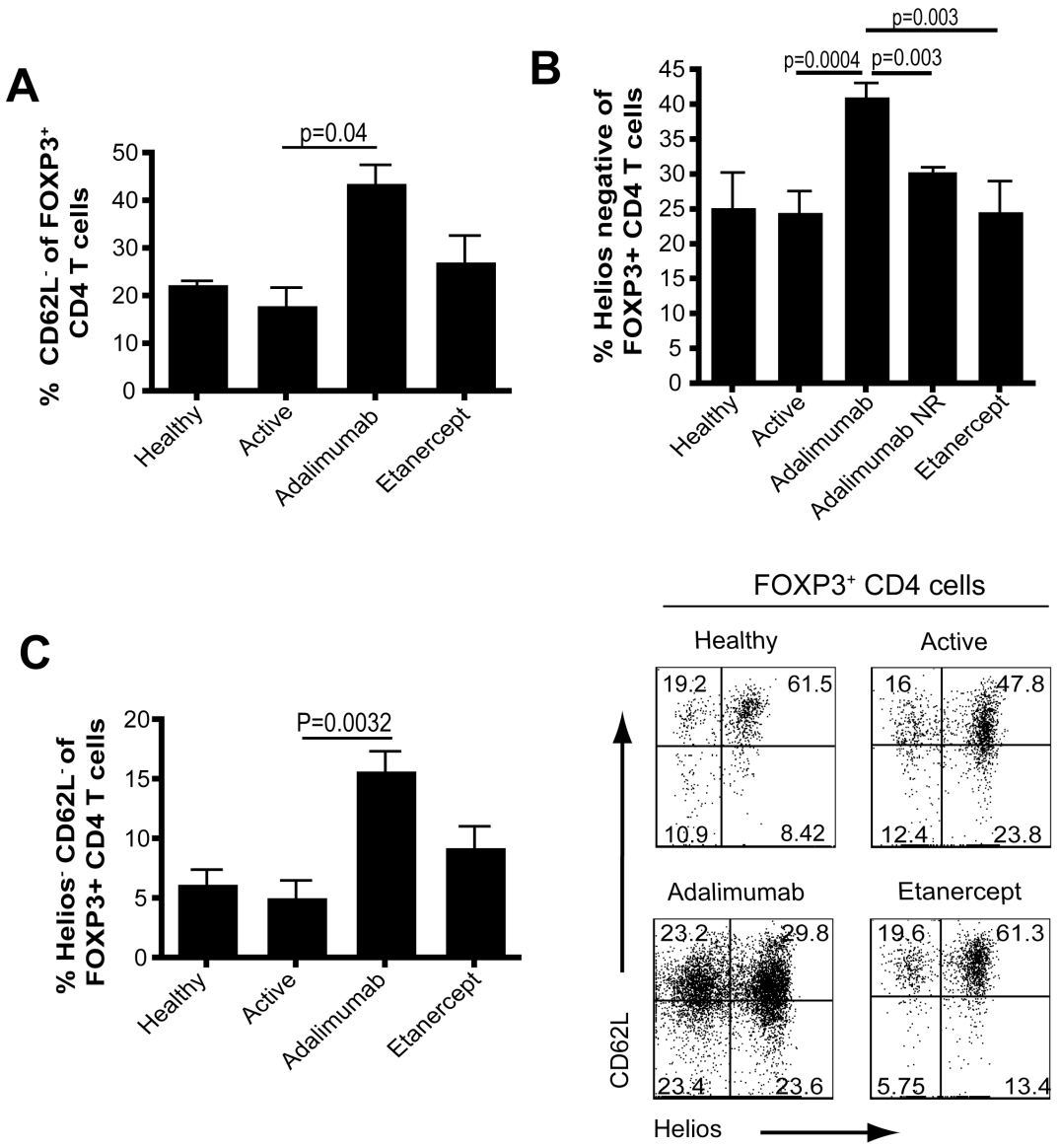


**Figure 3.3. Treg from patients treated with adalimumab bear the hallmarks of induced Treg.**

*Ex vivo* PBMC from healthy controls (*n=5*), patients with active RA (*n=10*) and patients responding to adalimumab (*n=8*) or etanercept (*n=8*) were stained with CD4, FOXP3, CD62L and Helios **A.** Percentage CD62L negative cells in the $CD4^+FOXP3^+$ population. **B**. Percentage Helios negative cells in the $CD4^+FOXP3^+$ population. Data from patients not responding to adalimumab are also shown (adalimumab NR) (*n=4*) **C.** Percentage Helios negative CD62L negative cells in the $CD4^+FOXP3^+$ population. Example FACS plots are gated on $CD4^+FOXP3^+$ cells and show co-staining of CD62L and Helios. For all graphs, bars represent the mean±SE.

### 3.3 Proliferation of FOXP3$^+$ T cells

In order to show that the increase in Treg in adalimumab treated patients was not a result of *in vivo* proliferation, cells were stained *ex vivo* for Ki67, an intracellular marker identifying cells that have recently proliferated. Treg show relatively high levels of Ki67, in line with data showing that Treg can proliferate substantially *in vivo* [423], however there were no differences in Treg proliferation observed between healthy controls and the patient groups studied (Figure 3.4 A).

### 3.4 Proliferation and cell death in rheumatoid arthritis

Proliferation and cell death often form an integral part of RA disease pathology due to the fundamental role cytokines such as TNF play in these processes [424]. Therefore, I examined proliferation and apoptosis in healthy controls and patients before and after anti-TNF therapy. There was no significant increase in CD4 T cell proliferation in patients treated with adalimumab compared to patients with active RA, healthy controls or patients responding to etanercept (Figure 3.4B).

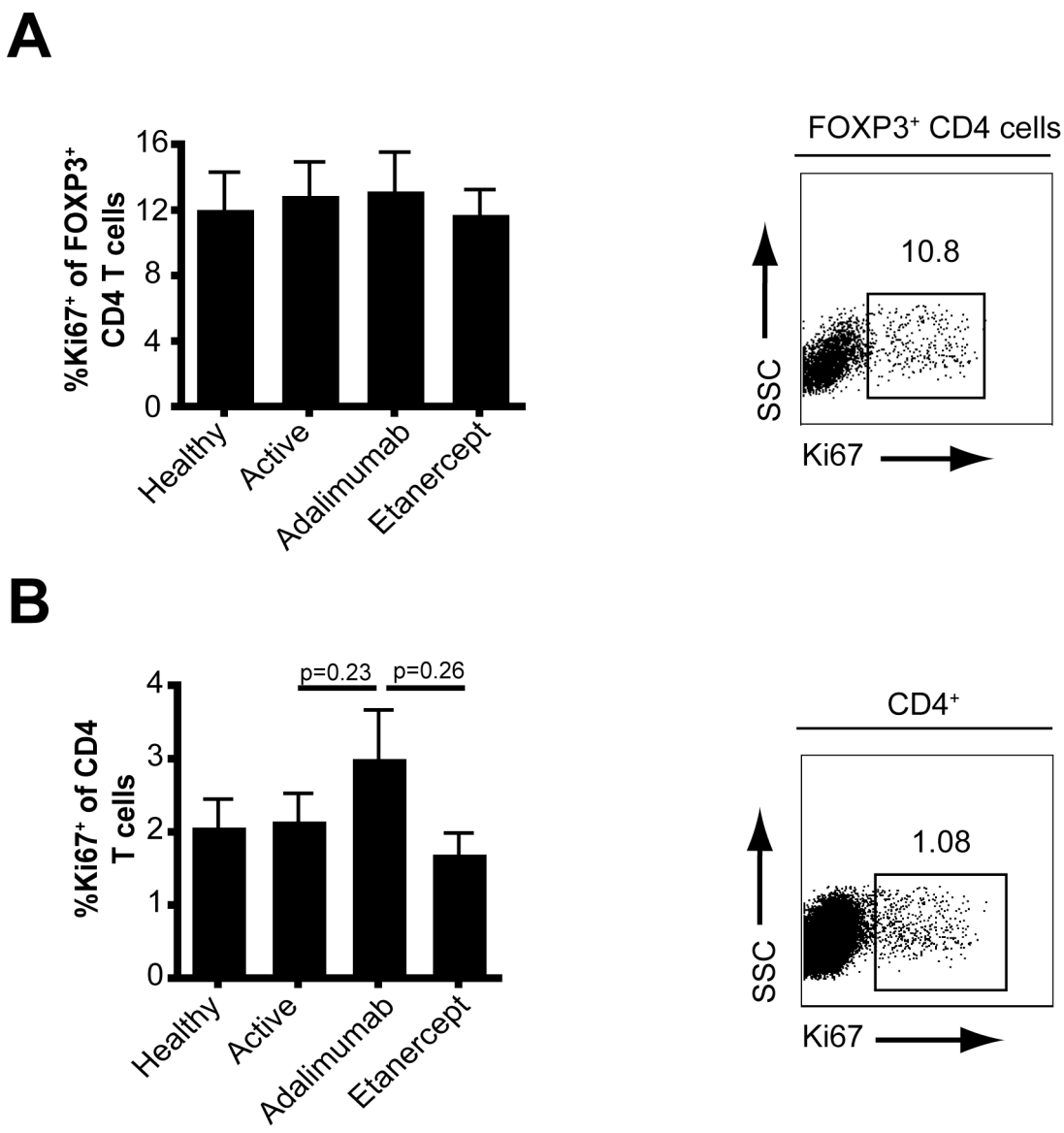


**Figure 3.4. CD4 cell proliferation in rheumatoid arthritis.**

*Ex vivo* PBMC from healthy controls (*n=7*), patients with active RA (*n=16*) and patients responding to adalimumab (*n=11*) or etanercept (*n=5*) were stained with CD4, FOXP3 and Ki67. **A.** Percentage $Ki67^+$ cells in the $CD4^+FOXP3^+$ population. An example FACS plot is shown. **B.** Percentage $Ki67^+$ CD4 cells, example FACS plot shown. For all graphs, bars represent the mean±SE.

To determine the percentage of dead/apoptotic cells present in patients, samples were stained for flow cytometry with Annexin V and 7AAD: the former is an anti-phospholipid binding protein which recognises phospholipids on the cell membrane during apoptosis whilst the latter is a vital dye, permeable only to cells which are dead or damaged. When whole PBMC were analysed there were no differences in early apoptotic cells (Annexin V+ $7AAD^-$) between healthy controls and the RA patients studied (Figure 3.5 A). There was a small reduction in dead and dying cells (Annexin $V^+7AAD^+$) in patients with active RA ($p=0.26$) compared to healthy controls, though this did not reach significance. Patients treated with adalimumab had similar levels of cell death as healthy controls, whilst patients treated with etanercept resembled more closely the level of cell death in patients with active RA (Figure 3.5 B).

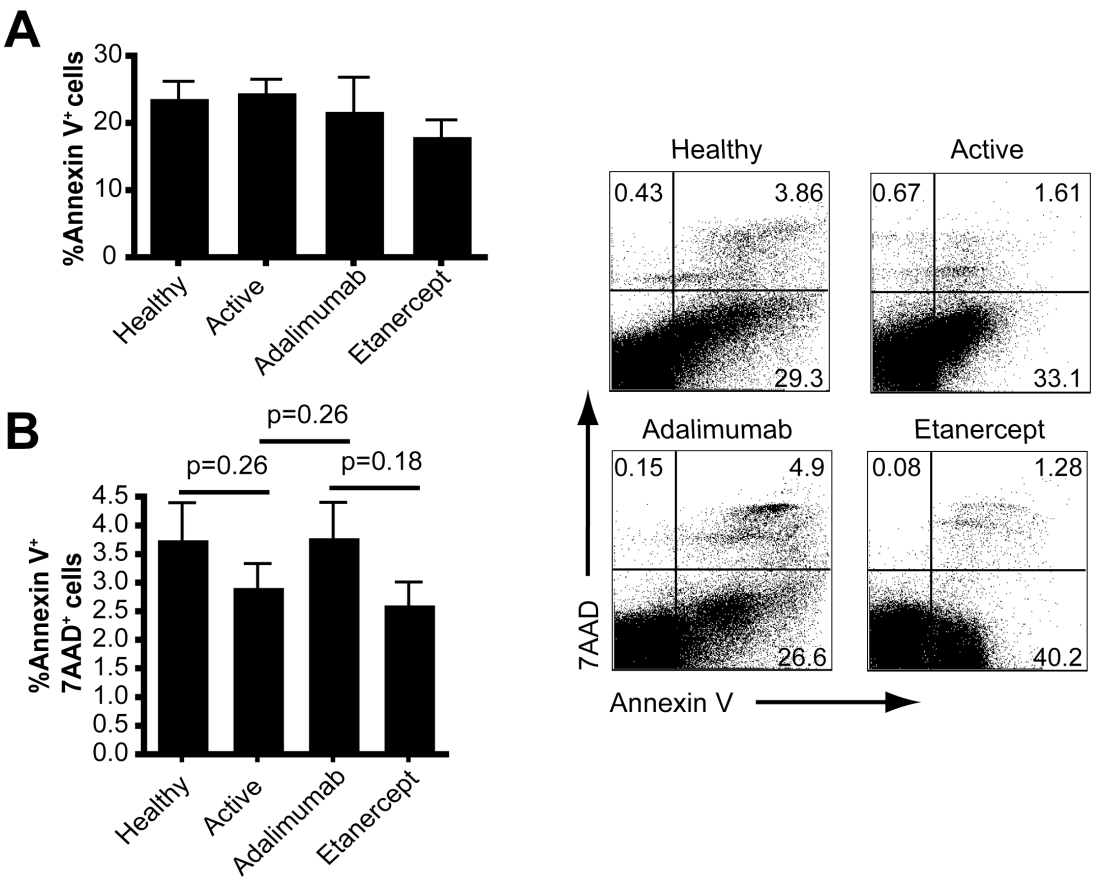


**Figure 3.5. Apoptosis and cell death in rheumatoid arthritis.**

Ex vivo PBMC were stained with Annexin V and 7AAD. A 'live' gate was not used for analysis **A**. Percentage Annexin $V^+$ cells (healthy n=10, active n=16, adalimumab n=6, etanercept n= 3). **B.** Percentage Annexin $V^+7AAD^+$ cells (healthy n=10, active n=17, adalimumab n=7, etanercept n= 5). Representative FACS plots are shown**.**

It has previously been shown that monocyte cell death is reduced in patients with active RA compared to healthy controls and that this is reversed by *in vitro* treatment with infliximab [425]. Indeed, our data showed a significant reduction in monocyte cell death in patients with active RA compared to healthy controls ($p=0.0006$). Moreover, there was a trend for levels of cell death to be restored to normal levels in patients treated with adalimumab ($p=0.12$), but not in patients treated with etanercept (Figure 3.6). These data may reach significance if the 'n' number is increased.

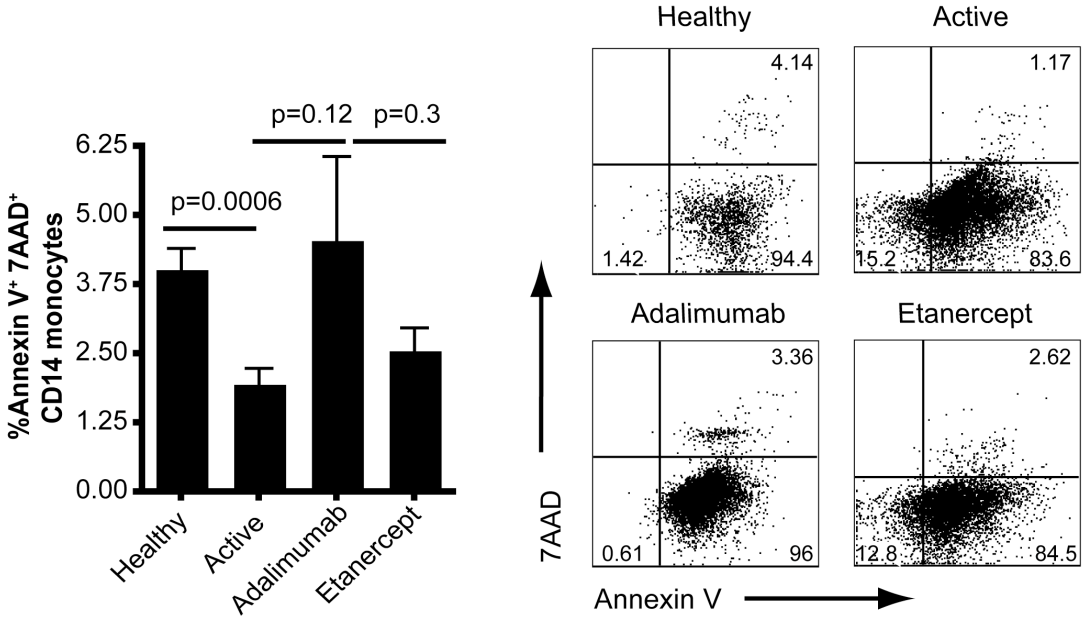


**Figure 3.6. Monocyte death in rheumatoid arthritis.**

Percentage Annexin$^{+}$7AAD$^{+}$ cells in the CD14$^{+}$ population. (healthy *n=9*, active *n=14*, adalimumab *n=6*, etanercept *n= 4*). A 'live' gate was not used for analysis. For all graphs, bars represent the mean±SE. Representative FACS plots are shown.

### 3.5 TGF-β expression on CD4 T cells

Previously, this group has shown that $CD4^+CD25^-$ derived TGF-β is important for the induction of $FOXP3^+$ cells in the presence of infliximab [86]. Thus we examined the expression of TGF-β on $FOXP3^+$ and $FOXP3^-$ CD4 T cells. There was no significant increase in the TGF-β expression on the surface of either $FOXP3^+$ or $FOXP3^-$ T cells from adalimumab treated patients compared to patients with active RA or patients treated with etanercept (Figure 3.7 A and B). However, some etanercept treated patients had high levels of TGFβ expression, so increasing the 'n' numbers of this study may reveal a significant increase in etanercept treated patients.

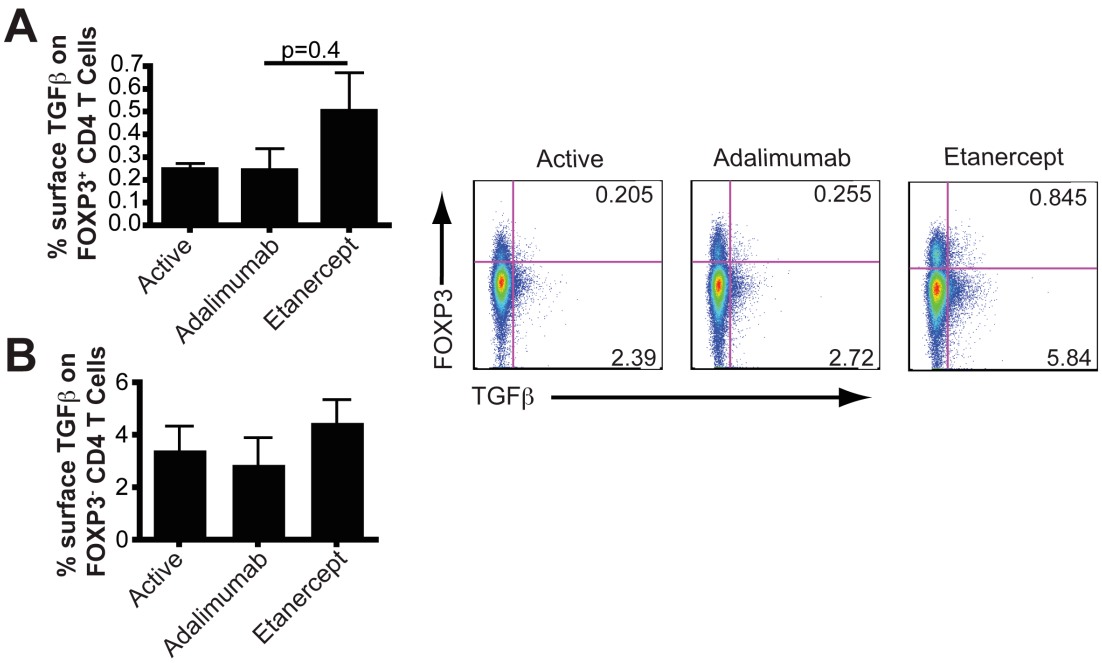


**Figure 3.7. Expression of TGF-β on CD4 T cells.**

PBMC from patients with active RA and patients treated with adalimumab or etanercept (*n=4*) were thawed and surface stained for CD4 and TGF-β, then stained for intracellular FOXP3. **A.** Percentage of surface TGF-β in the $FOXP3^+$ population. **B.** Percentage of surface TGF-β in the $FOXP3^-$ population. Example FACS plots are gated on CD4. For all graphs, bars represent the mean±SE.

### 3.6 IL-10 production from CD4 T cells

In addition to TGF-β, IL-10 has been shown to be important for the induction of Treg [426, 427], so we examined IL-10 production by T cells, B cells and monocytes from peripheral blood. When we examined CD4 T cells, there were no significant differences in IL-10 production between patient groups (Figure 3.8 A). The source of IL-10 from CD4 T cells for patients with active RA and those treated with adalimumab appeared to be $FOXP3^-$ cells. Very little IL-10 was detectable from the $FOXP3^+$ cells by FACS (Figure 3.8 B).

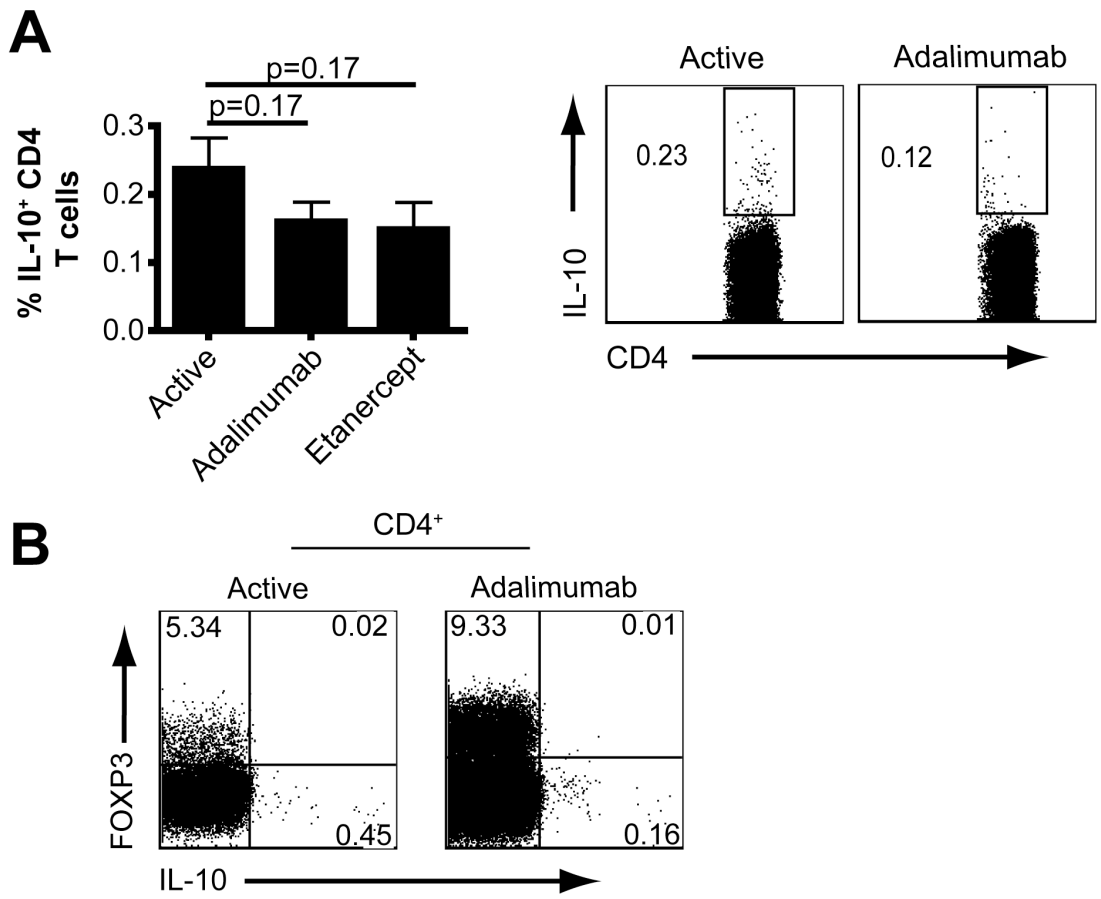


**Figure 3.8. IL-10 production from CD4 T cells.**

PBMC from patients with active RA and patients treated with adalimumab or etanercept were thawed and stained for CD4 before stimulation with PMA for 4 hours and subsequent staining with IL-10 (*n=6*). **A**. Percentage IL-10$^+$ T cells. Bars represent the mean±SE. Example FACS plots shown for an active patient and an adalimumab patient. **B.** Example FACS plots gated on CD4, show IL-10 staining from FOXP3$^+$ and FOXP3$^-$ populations in a patient with active RA and a patient treated with adalimumab.

### 3.7 IL-10 production from B cells

In contrast to the data from CD4 T cells, there was a trend for IL-10 production from $CD19^+$ B cells to increase after adalimumab therapy (p=0.07) but not etanercept therapy (p=0.22) (Figure 3.9 A). $CD19^+$ cells were analysed based on their expression of CD38 and CD24. Using previous publications as a guideline [428], 3 distinct populations of B cells were identified; $CD24^{hi}$ $CD38^-$ cells were classified as memory B cells, $CD24^{int}CD38^{int}$ were classified as mature B cells and $CD24^{hi}$ $CD38^{hi}$ were classified as immature B cells. This gating strategy was then applied to the $CD19^+IL\text{-}10^+$ cells to determine which subsets were contributing to total B cell IL-10 (Figure 3.9 B). It appeared that the greatest proportion of IL-10 came from the mature subset, with less coming from the immature subset and very little from the memory cell population (Figure 3.9 C). Whilst patients with active RA and patients treated with adalimumab had similar levels of IL-10 producing cells from the mature B cell subset, etanercept treated patients possessed significantly more $IL\text{-}10^+$ mature B cells compared to patients treated with adalimumab (Figure 3.9 C). Indeed, despite low 'n' numbers for the active RA cohort there was still a an increase in $IL\text{-}10^+$ mature B cells in etanercept patients compared to patients with active RA which may reach significance if more patients were studied (Figure 3.9 C). There were no significant differences between any of the patient groups in percentage of IL-10 producing cells in the immature subset (Figure 3.8 D).

As IL-10 production from immature B cells has been shown to be important to Treg maintenance and stability [257], IL-10 production was analysed from a different perspective. Rather than examining how B cell subsets contributed to total B cell IL-10, the percentage of immature cells that make IL-10 was determined. Whilst it

appeared that immature B cells from adalimumab treated patients made more IL-10 than their counterparts in active patients or those treated with etanercept, these differences were not significant (Figure 3.9E).

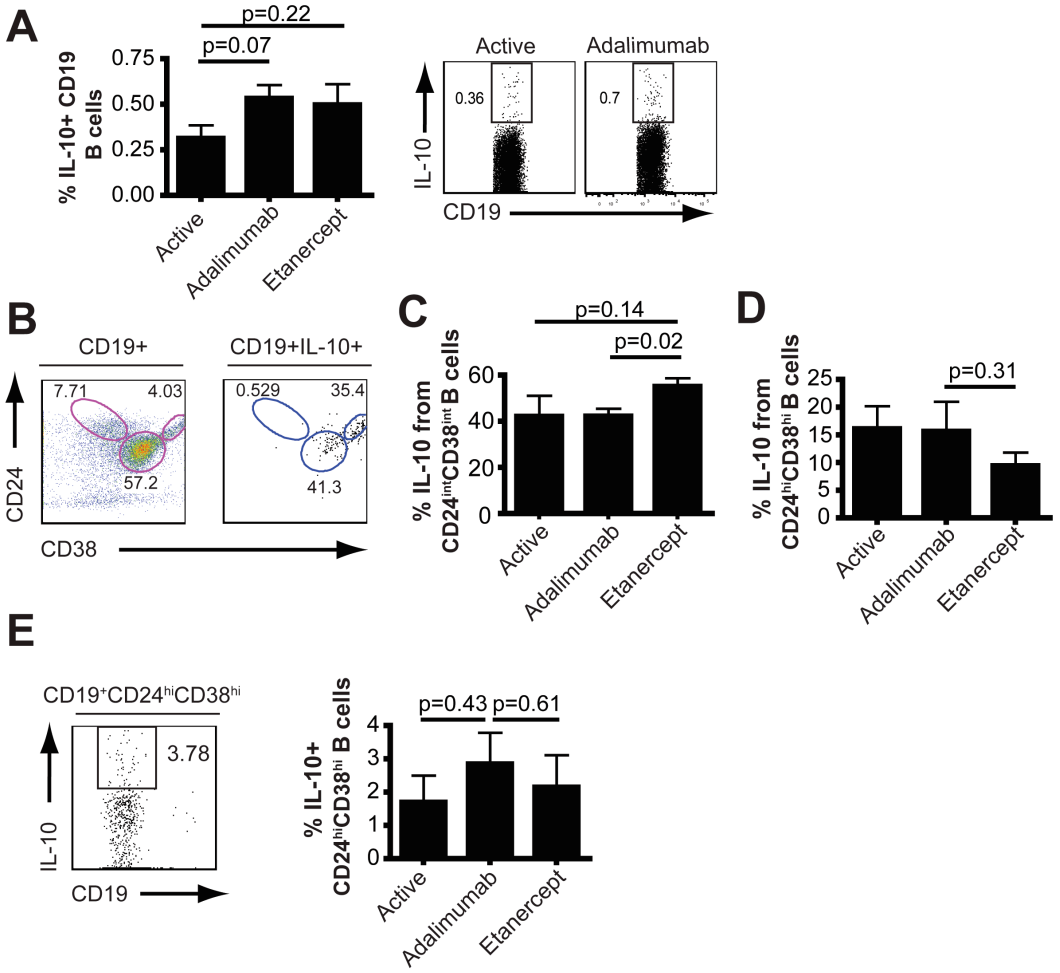


**Figure 3.9. IL-10 production from B cells.**

PBMC from patients with active RA and patients treated with adalimumab or etanercept were thawed and stimulated with PMA for 4 hours before staining for B cell markers and IL-10 (*n=6*). **A**. Percentage IL-$10^+$ $CD19^+$ cells. Bars represent the mean±SE. Example FACS plots shown for an active RA patient and an adalimumab treated RA patient**. B**. Cells were stained for CD19, CD24 and CD38. The left FACS plot is gated on $CD19^+$ cells and the right is gated on IL-$10^+$ cells. In both cases, cells were divided into 3 populations: $CD24^{hi}$ $CD38^-$ (memory), $CD24^{int}CD38^{int}$ (mature) and $CD24^{hi}$ $CD38^{hi}$ (immature). **C**. Percentage of total B cell IL-10 produced by mature B cells. Bars represent the mean±SE. **D.** Percentage of total B cell IL-10 produced by immature B cells. Bars represent the mean±SE **E**. FACS plot shows an example of IL-10 production from immature B cells. The graph shows pooled data of percentage IL-10 production from immature B cells. Bars represent the mean±SE.

### 3.8 IL-10 production from CD14$^+$ monocytes

Finally, we analysed the IL-10 production from CD14$^+$ monocytes. Little IL-10 was detected from this subset but it seemed as though there was more IL-10 production from monocytes in the etanercept treated patient group than patients with active RA or patients treated with adalimumab (Figure 3.10) however, this did not reach significance. Increasing the power of this study may reveal significance or further analysis may reveal that etanercept treated RA patients fall into distinct groupings which may explain the variability observed.

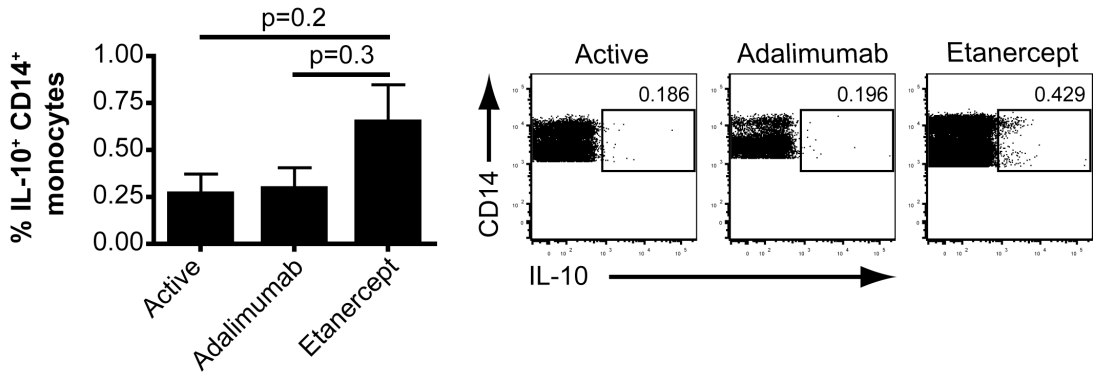


**Figure 3.10. IL-10 production from monocytes.**

PBMC from patients with active RA and patients treated with adalimumab or etanercept were thawed and stimulated with PMA for 4 hours before staining with CD14 and IL-10 (*n=6*). Graph shows pooled data of percentage IL-10$^+$ monocytes. Bars represent the mean±SE. Example FACS plots shown.

### 3.9 Retinoic-acid-receptor-related orphan receptors identify Th17 cells

Previous studies have shown that Th17 cells are increased in the periphery of patients with active RA [208]. In addition, it is now widely accepted that Th17 cells and Treg exist on a developmental axis [429]. In this report, I have shown that patients treated with adalimumab have increased peripheral Treg (Figure 3.1). Therefore, it was investigated whether these changes in Treg led to reciprocal changes in Th17 cells in the periphery. It has recently become possible to detect ROR proteins, the master transcription factors of Th17 cells, by FACS using a rat antibody specific for the human and mouse ROR homologues. Initial optimisation was undertaken using cells of harvested lymph nodes from mice immunised with methylated BSA (see methods section 2.4). These cells were co-stained for RORγt and IL-17 after 4 hours of stimulation and analysed by FACS. More than 90% of the IL-17 produced was from cells that expressed RORγt, identifying them as Th17 cells (Figure 3.11 A). When this was translated into samples from human peripheral blood, it was clear that IL-17 producing T cells were within the human $RORC^{+}$ population (Figure 3.11 B). This confirmed the utility of this antibody in identifying IL-17 producing cells or their precursors.

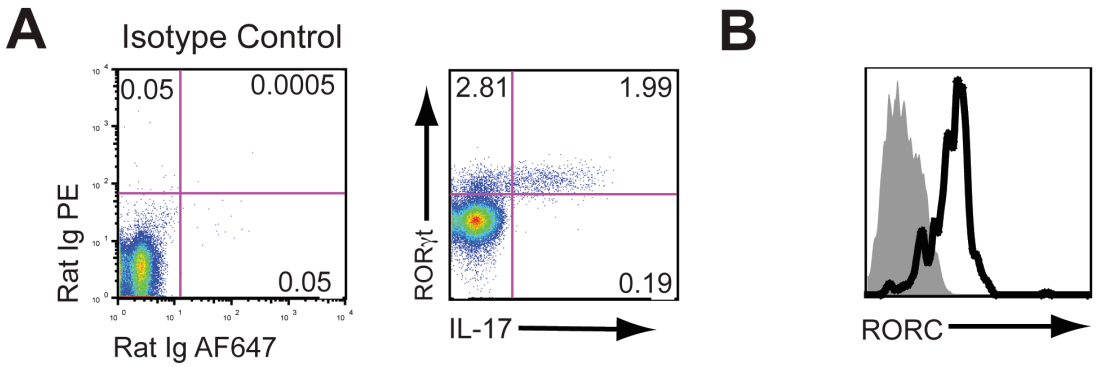


**Figure 3.11. RORγt as a marker of Th17 cells.**

**A.** Cells from the lymph node of an mBSA-immunised mouse were stimulated for 4 hours with PMA, ionomycin and Golgi stop. Cells were then stained for CD4, fixed and incubated with an Fc block before the addition of antibodies for RORγt and IL-17. The isotype control was used to determine gating strategy. **B.** Histogram showing expression of human RORC. PBMC from a healthy control were stimulated for 4 hours with PMA, ionomycin and Golgi stop. Cells were then stained for CD4, fixed and incubated with an Fc block, before the addition of antibodies against RORC and IL-17. The solid grey histogram is the isotype control for RORC and the outline is gated on the IL-17$^{+}$ cells

### 3.10 Th17 cells are reduced in patients treated with adalimumab, but not etanercept.

Using the approach described in 3.8, we explored whether the increase in FOXP3 expression in adalimumab treated patients influenced the expression RORC, the human ROR homologue. The expression of RORC, was significantly elevated in active RA patients compared to healthy controls (*P =0.0007*) whereas $RORC^+$ T cells were reduced 2-fold in patients treated with adalimumab (*P=0.002*) but not etanercept. Moreover, levels of $RORC^+$ cells were significantly lower in patients responding to adalimumab than those patients who were not responding to therapy (*P=0.02*) (Figure 3.12 A). An increased ratio of $FOXP3^+$ cells to $RORC^+$ cells in adalimumab treated patients compared to any other group confirmed the shift towards an immunoregulatory T cell phenotype (Figure 3.12 B). These differences were less pronounced when IL-17 production was determined *ex vivo* (Figure 3.12 C). Finally, IFNγ production by CD4 T cells was examined because expression of this cytokine is not known to have an intrinsic relationship with Treg. Thus, as expected there were no significant differences in IFNγ production between healthy controls, patients with active RA and patients treated with adalimumab or etanercept (Figure 3.12 D).

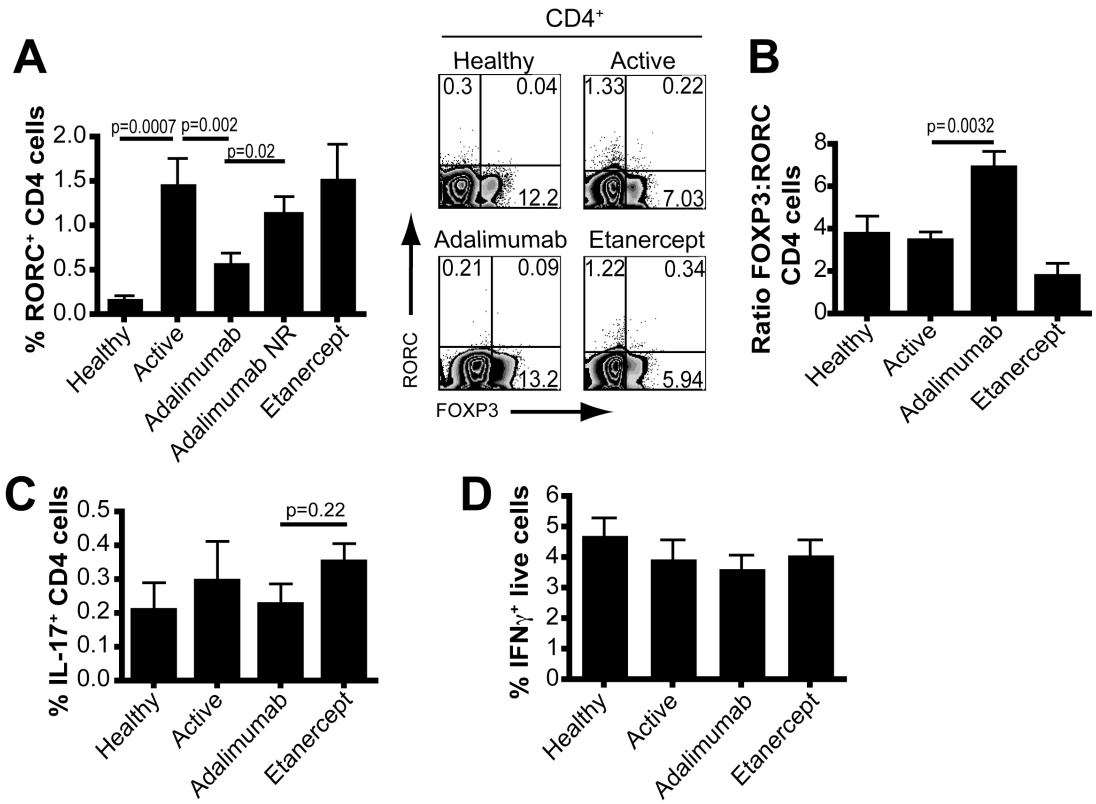


**Figure 3.12. Th17 cells are reduced in RA patients treated with adalimumab, but not etanercept.**

**A.** PBMC from healthy controls, patients with active RA, patients responding to adalimumab or etanercept were stained *ex vivo* for CD4, RORC and FOXP3. Data from patients not responding to adalimumab are also included (*n=4).* Bars indicate mean percentage $CD4^+RORC^+$ cells ±SE *(n=10)*. Representative FACS plots reveal co-staining of RORC and FOXP3 in $CD4^+$ T cells. **B.** Ratio of $RORC^+$ to $FOXP3^+$ CD4 T cells in the different patient groups and healthy controls. Bars represent mean ±SE **C.** *Ex vivo* PBMC were stimulated for 4 hours with PMA before staining with IL-17 (*n=6*). Graphs show percentage $CD4^+$ $IL\text{-}17^+$ cells. Bars represent mean ±SE **D.** *Ex vivo* PBMC were stimulated as in **'C'** and stained for IFNγ. Healthy *n=7*, active *n=17*, adalimumab *n=19*, etanercept *n=7*.Graphs show percentage $IFN\gamma^+$ cells. Bars represent mean ±SE

Summary:

In confirmation of previously published data, this chapter has shown that there is no difference in the percentage of circulating Treg in healthy controls and patients with active RA. Like patients treated with infliximab, patients responding to adalimumab demonstrated an increase in Treg but this was absent in RA patients responding to etanercept. Response to adalimumab therapy was vital for this increase in Treg because levels of Treg in patients that failed to respond to therapy were similar to patients with active RA and those treated with etanercept. Examination of Treg phenotype showed no evidence of increased proliferation of Treg from adalimumab treated patients compared to patients with active RA. Indeed, a greater percentage of Treg from adalimumab treated patients bore markers of induced Treg than etanercept treated patients or patients with active RA, suggesting an extra-thymic source.

Compared to healthy controls, overall levels of apoptosis were lower in patients with active RA. The data show differences between apoptosis in adalimumab and etanercept treated patients but these do not reach significance. Examination of anti-inflammatory cytokines revealed few significant differences between patients with active RA and those responding to adalimumab. Though some etanercept treated patients had high levels of TGFβ and IL-10 production compared to other patient groups. Examination of changes in inflammatory T helper cell subsets showed that $RORC^{+}$ Th17 cells were elevated in patients with active RA compared to healthy controls and reduced by adalimumab but not etanercept therapy. These differences were not as stark when IL-17 production by T cells *ex vivo* was determined. There were no differences in T cell IFNγ production in any of the patient groups studied.

# Chapter 4

## Regulatory T cell function in rheumatoid arthritis patients treated with anti-TNF

Objectives:

1. Using well-established suppression assays, confirm a defect in the capacity of Treg from patients with active RA to suppress IFNγ and determine if Treg from adalimumab treated RA patients demonstrate a restored capacity to suppress this cytokine.
2. Demonstrate, using the same approach, that the absence of induced Treg in etanercept treated RA patients is associated with no restoration of suppressive function.
3. Use a method where whole PBMC are depleted of Treg to show that Treg from adalimumab treated patients, but not etanercept treated patients regulate Th17 cells.
4. Confirm the capacity of Treg to control Th17 cells with co-culture experiments.
5. Elucidate the mechanism of Treg suppression of IL-17 using anti-IL-10 and anti-TGFβ blocking antibodies in suppression assays.

## 4.1 Treg from RA patients treated with adalimumab, but not etanercept, can suppress IFNγ production via the production of IL-10 and TGF-β

Our previous publications have shown that Treg from patients with active RA (RATreg) are defective in their suppression of Th1 responses [124]. This defect was reversed by infliximab therapy via the induction of Treg. Thus, it was hypothesised that Treg from patients treated with adalimumab (AdTreg) would be able to suppress IFNγ, but due to the absence of Treg induction in etanercept treated patients, Treg from these patients (EtTreg) would remain defective. As previously published, Treg from healthy controls (HTreg) were able to suppress IFNγ, and this ability was reduced in RATreg. AdTreg showed equivalent suppressor ability to their healthy counterparts and as predicted, EtTreg remained defective. In accordance with data previously published by this group [86], neutralization of IL-10 and TGF-β reversed the ability of AdTreg to suppress IFNγ (Figure 4.1 A). Furthermore, examination of IL-10 in the supernatants of these cultures showed that IL-10 increased upon the addition of AdTreg to autologous responder T cells suggesting that AdTreg but not RATreg or EtTreg produce IL-10 (Figure 4.1 B).

As TGF-β plays such an intrinsic role in the suppression of IFNγ by AdTreg, we investigated if CD4 T cells from patients treated with adalimumab have increased expression of the receptor for TGF-β. A number of adalimumab treated patients demonstrated high levels of TGF-βRII expression on both the $FOXP3^+$ (Figure 4.1 C) and $FOXP3^-$ (Figure 4.1 D) CD4 cells. This did not reach significance but these data may reveal a difference with sufficient power.

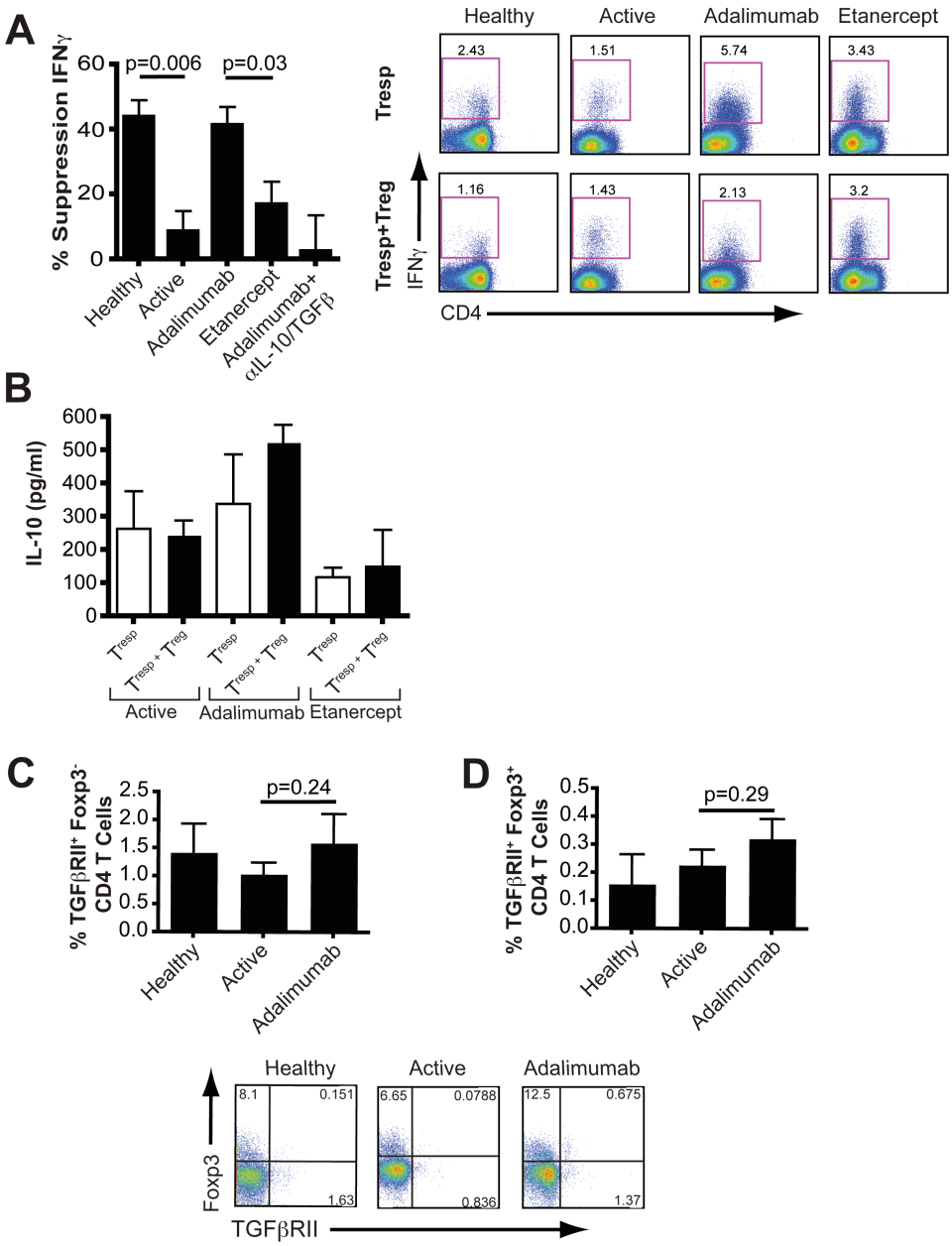


**Figure 4.1. Treg from patients treated with adalimumab, but not etanercept can suppress IFNγ via the production of IL-10 and TGF-β.**
**A.** $CD4^+CD25^+CD127^-$ cells (Treg) and $CD4^+CD25^-CD127^+$ cells (Tresp) were isolated from PBMC and cultured in a ratio of 3 Tresp to 1 Treg and stimulated with soluble anti-CD3 and anti-CD28 for 4 days before re-stimulating cells with PMA, ionomycin and Golgi Stop for 4 hours. Bars represent mean suppression of IFNγ (*n=5*). Representative FACS plots show IFNγ production from responder T cells in cultures with or without Treg. **B.** Supernatants from the co-culture described in ***A.*** were assayed for IL-10 (*n=3*) **C-D**. PBMC from healthy controls (*n=4*), patients with active RA (*n=24*) and patients responding to adalimumab (*n=13*) were stained for CD4, TGF-β receptor II and FOXP3. **C.** Percentage of surface TGF-βRII in the $FOXP3^-$ population. **D.** Percentage of surface TGF-βRII in the $FOXP3^+$ population. Example FACS plots are gated on CD4. For all graphs bars represent the mean±SE.

## 4.2 Treg from patients treated with adalimumab, but not patients treated with etanercept suppress IL-17 from whole PBMC

IL-17 has been implicated in the progression of a number of autoimmune diseases and has been detected in the joints of patients with RA [430]. In contrast to their ability to suppress responder T cell proliferation or IFNγ production, this lab and others have shown that both HTreg and RATreg are unable to suppress IL-17 production *in vitro* [37, 194]. This report has detailed an increase in AdTreg concomitant with a reduction in Th17 cells; this led me to investigate if Treg induced by adalimumab therapy could suppress Th17 cells. In early co-culture experiments, IL-17 production was negligible from responder T cells cultured alone. It is known that Toll-like receptor-activated monocytes can drive IL-17 production from T cells [194]. Thus, an approach was used where Treg were depleted from whole PBMC so that monocytes were present in the culture to promote Th17 responses. However, I chose not to stimulate PBMC with TLR agonists in order to more closely reflect the *in vivo* environment where monocytes may already have been activated. Cells were sorted into two populations, one sample of whole PBMC and one sample of PBMC depleted of $CD4^+CD25^+CD127^-$ Treg (for purity see Figure 2.2). These were cultured for 3 days with anti-CD3 and anti-CD28, and IL-17 secretion was measured by FACS and ELISA.

The percentage difference in IL-17 production between whole PBMC and PBMC-Treg was calculated. Consistent with previously published data [37], neither HTreg nor RATreg showed evidence of suppressing IL-17 as IL-17 did not significantly alter when Treg were depleted from these samples. However, IL-17 production increased when adalimumab patient samples were depleted of Treg, suggesting that AdTreg can suppress IL-17. In accordance with the data reported here suggesting

etanercept does not directly affect Treg numbers or function, EtTreg showed no evidence of suppressing IL-17 (Figure 4.2 A). These results were confirmed by measuring IL-17 using an ELISA assay, showing that IL-17 was only increased significantly ($p=0.001$) when the PBMC of adalimumab treated patients are depleted of Treg (Figure 4.2 B).

### 4.3 Treg from patients with active RA are unable to suppress IL-22 and this capacity is restored by treatment with adalimumab, but not etanercept

IL-22 is a cytokine known to be produced by Th17 cells [431] and similarly to IL-17, has been identified in the synovium of patients with active RA [432]. However, a recent paper has suggested that IL-22 can be anti-inflammatory during acute liver inflammation [308]. I therefore assayed IL-22 in PBMC cultures depleted of Treg. In contrast to their inability to suppress IL-17 production, HTreg were capable of suppressing IL-22. RATreg had little capacity to suppress IL-22, whilst AdTreg had a restored capacity to suppress this cytokine ($p=0.0159$) and this was absent in EtTreg (Figure 4.2 C).

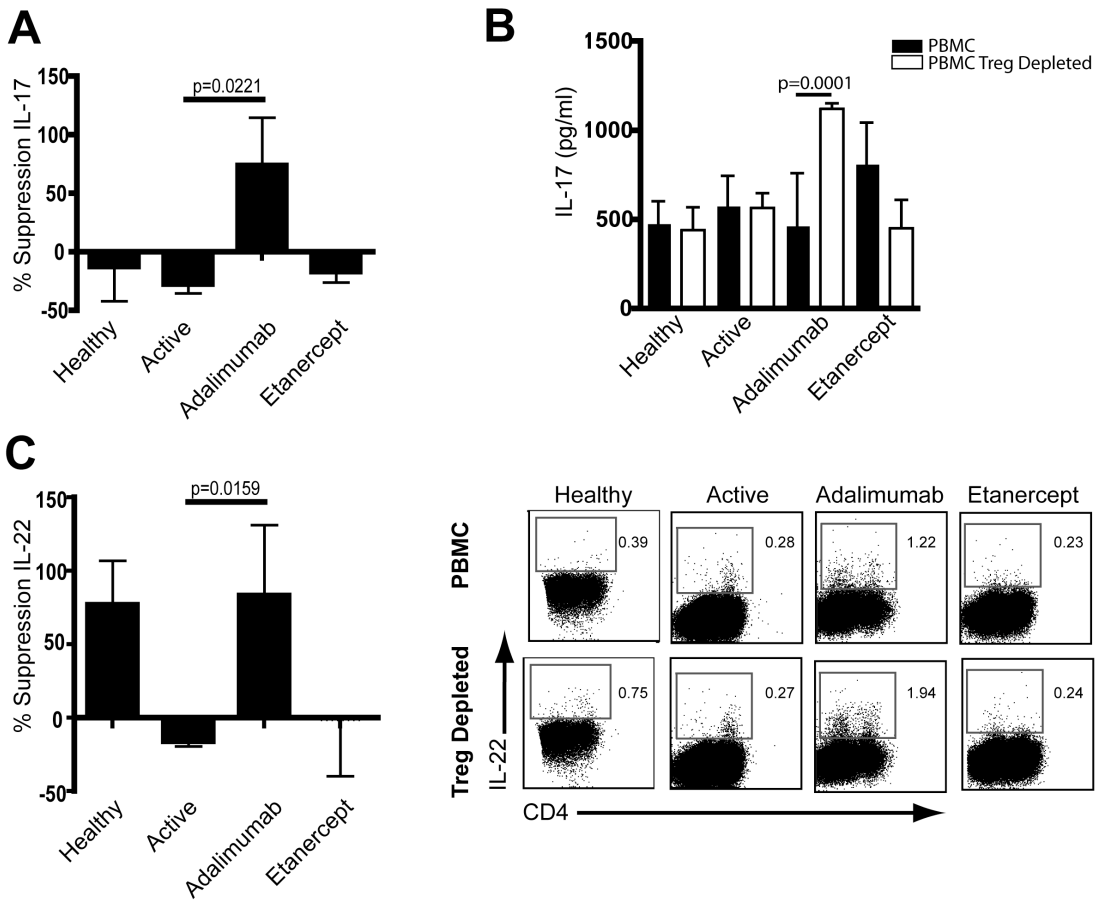


**Figure 4.2. Treg from RA patients treated with adalimumab, but not etanercept, are capable of suppressing IL-17 and IL-22 *in vitro*.**

**A.** PBMC were separated into 2 fractions, untouched PBMC and PBMC depleted of $CD4^+CD25^+CD127^-$ Treg. Cells were cultured for 3 days with soluble anti-CD3 and anti-CD28, and then stimulated with PMA, ionomycin and Golgi Stop. Cells from healthy controls (*n=5*), patients with active RA (*n=6*) and patients responding to adalimumab (*n=6*) or etanercept (*n=4*) were stained for IL-17. Bars represent the mean of percentage difference in IL-17 production between PBMC and PBMC depleted of Treg. **B.** Supernatants from ***A.*** were assayed for IL-17 secreted during culture using ELISA. Bars represent the mean±SE. **C.** PBMC or PBMC-Treg from healthy controls (*n=2*), patients with active RA (*n=6*) and patients responding to adalimumab (*n=4*) or etanercept (*n=4*) were stained with flow cytometry antibodies for IL-22. Bars represent the mean of the percentage difference in IL-22 production between PBMC and PBMC depleted of Treg. Representative FACS plots show IL-22 production from cultures of whole PBMC with (PBMC) or without (Treg depleted) Treg.

## 4.4 Treg from patients treated with adalimumab can suppress IL-17 production from CD4 T cells

In order to confirm that CD4 Treg were suppressing Th17 cells and not another IL-17 producing cell in whole PBMC, we co-cultured responder T cells in a 3:1 ratio with Treg, and to drive Th17 cells we added autologous $CD14^+$ monocytes. Monocytes were stained with a cell membrane dye (PKH) before culture so that they could be excluded from analysis. The addition of monocytes to responder T cells was sufficient to drive IL-17 and enhance IFNγ production from T cells (Figure 4.3 A). However, the addition of monocytes to this co-culture system did not reduce the capacity of HTreg or AdTreg to suppress IFNγ, nor alter the defect in RATreg or EtTreg (Figure 4.3 B).

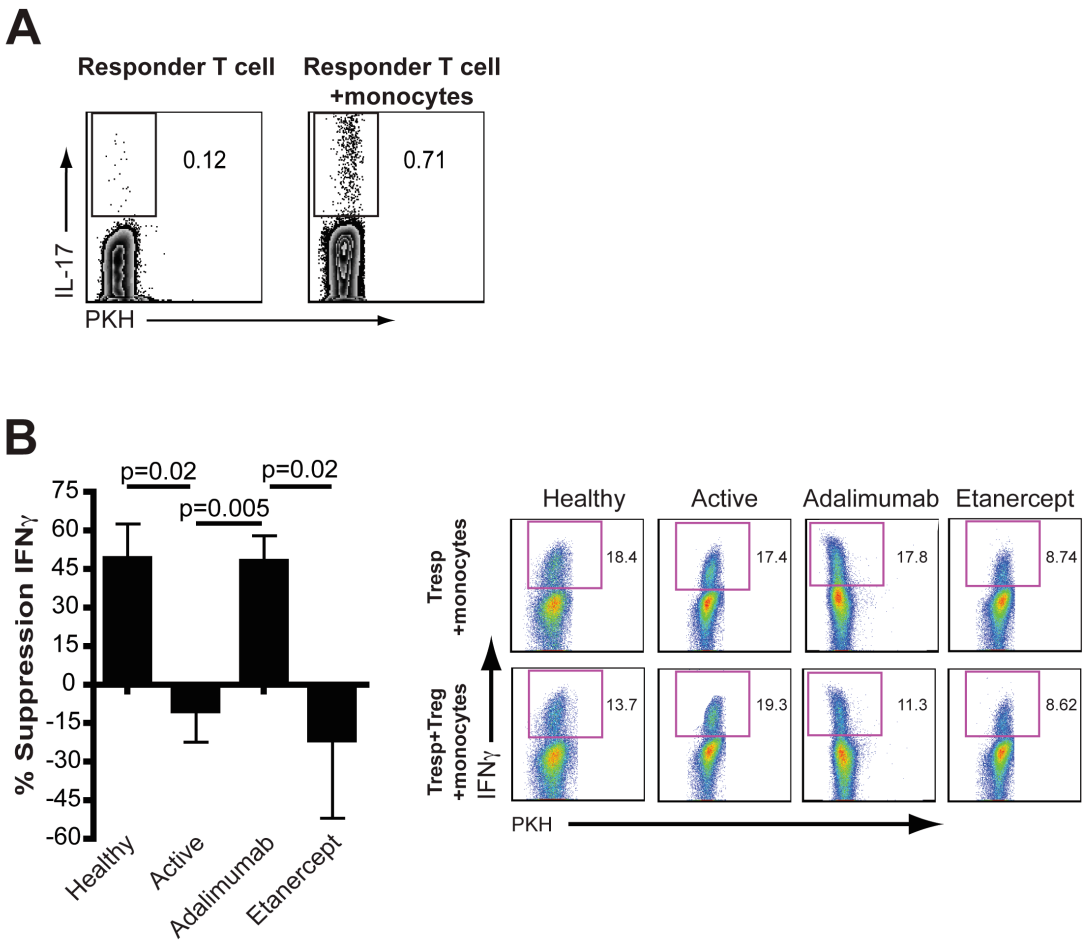


**Figure 4.3. Monocytes drive Th17 responses but do not alter Treg function.**

**A.** Responder T cells (Tresp) from adalimumab treated patients were cultured with 1µg/ml anti- CD3/28, alone or in the presence of PKH-stained monocytes for 5 days. FACS plots indicate IL-17 production from the PKH negative population. **B.** Treg, Tresp and monocytes were isolated and cultured with anti-CD3/CD28. Bars represent mean suppression of IFNγ ±SE. Healthy *(n=4)*, Active *(n=4)*, Adalimumab *(n=6)*, Etanercept *(n=4)*. Representative FACS plots show IFNγ production from cultures of responder T cells and monocytes with or without Treg.

Using this approach to investigate the suppression of IL-17, it was confirmed that only AdTreg could suppress IL-17 from responder T cells. IL-17 production either stayed the same or increased upon the addition of HTreg, RATreg or EtTreg. Moreover, Treg isolated from patients not responding to adalimumab did not acquire the capacity to suppress IL-17 (Figure 4.4 A). Assaying IL-17 in the culture supernatants revealed that its production fell only when AdTreg were added to their autologous responder T cells and monocytes (Figure 4.4 B). Of interest, there was more IL-17 present in the supernatants of responder T cells and monocytes from adalimumab treated patients, compared to the other patient groups and healthy controls. This was consistent with the data presented in Figure 4.2 B, where the highest IL-17 production was detected in PBMC depleted of Treg from adalimumab treated patients.

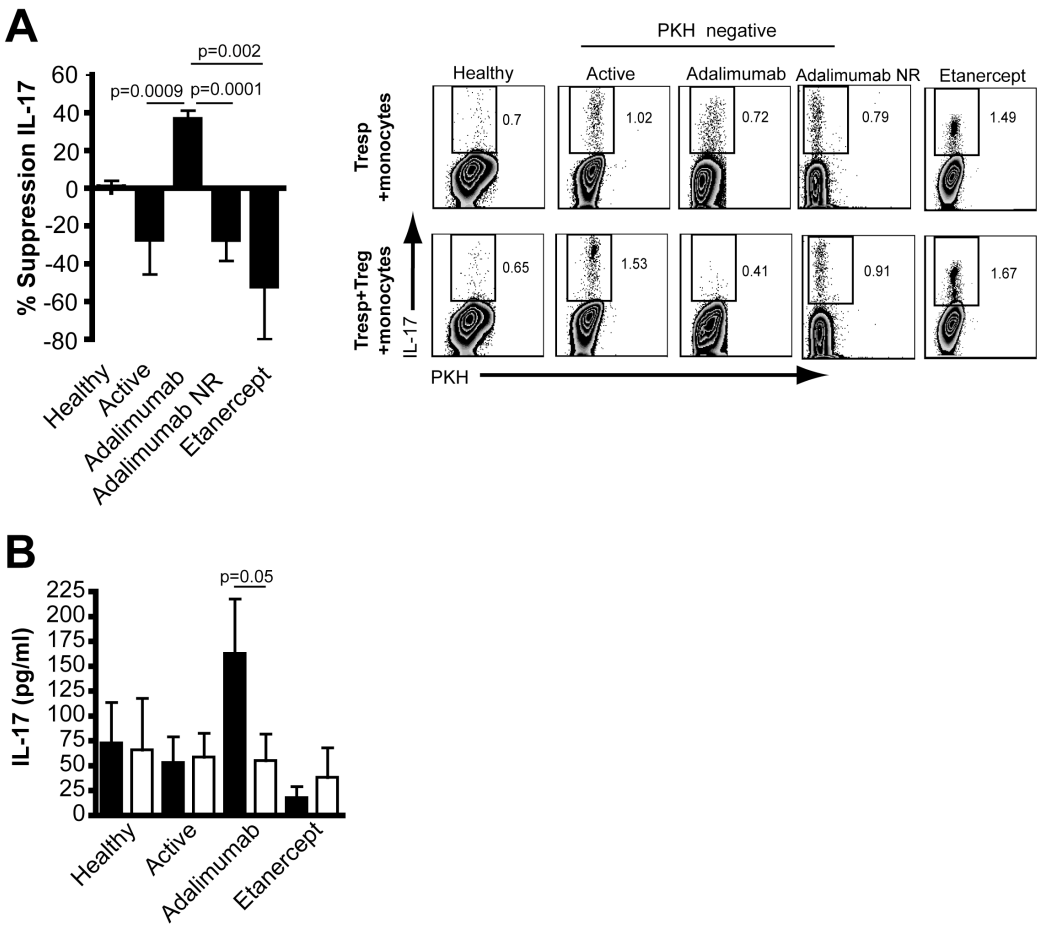


**Figure 4.4. Treg isolated from RA patients treated with adalimumab can suppress Th17 cells.**

**A.** Treg, responder T cells (Tresp) and monocytes from healthy controls, patients with active RA and patients responding to adalimumab or etanercept were isolated and cultured with anti-CD3/CD28 *(n=10)*. Data are also shown for patients not responding to adalimumab (adalimumab NR) (*n=3*) Bars represent mean suppression of IL-17 ±SE. Representative FACS plots show IL-17 production from responder T cells co-cultured with monocytes with or without Treg. Monocytes were excluded from analysis by PKH staining **B.** Supernatants from ***A.*** were analysed for IL-17 by CBA. Bars represent the mean±SE.

## 4.5 Suppression of IL-17 is independent of IL-10 and TGF-β

Next we used neutralising antibodies to IL-10 and TGF-β to investigate the role of these cytokines in the suppression of IL-17. In accordance with previously published data using infliximab treated patients [86], the blockade of IL-10 alone, TGF-β alone, or both TGF-β and IL-10 was able to significantly reduce the ability of AdTreg to suppress IFNγ. In contrast, blockade of IL-10 and TGF-β had no effect on the ability of Treg from adalimumab treated patients to suppress IL-17, indicating that these Treg control IFNγ and IL-17 through distinct mechanisms (Figure 4.5 A).

To provide further evidence that that IL-10 signalling was not mediating the AdTreg suppression of IL-17, an anti-IL-10 receptor antibody alone or in conjunction with TGF-β and IL-10 neutralising antibodies was added to the cultures. Under none of these conditions was the ability of AdTreg to suppress IL-17 reversed (Figure 4.5 B).

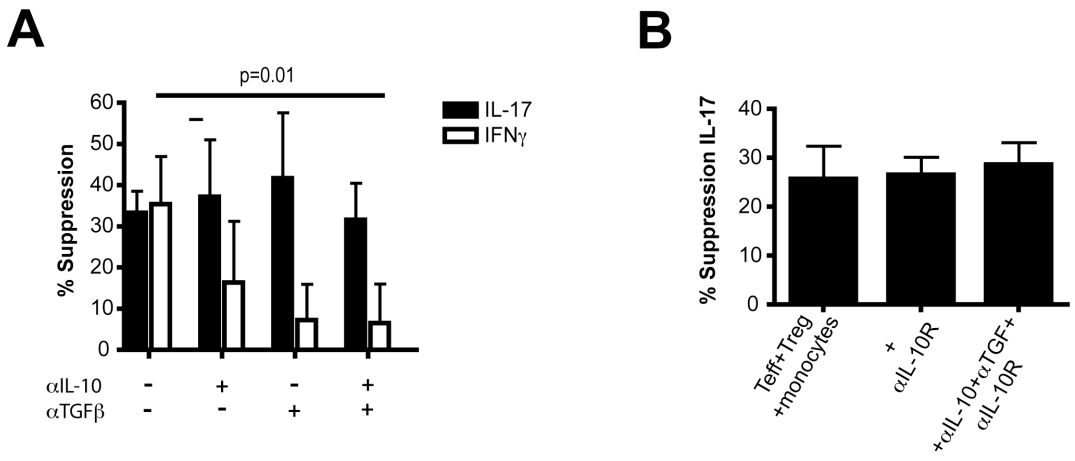


**Figure 4. 5. Suppression of IL-17 by AdTreg is independent of IL-10 and TGF-β**
The suppression assay described in Figure 4.4 was repeated with adalimumab patient samples **A.** Neutralising antibodies to IL-10 and TGF-β were added to cultures. Cells were stained for IL-17 and IFNγ. Bars represent mean suppression of IL-17 or IFNγ *(n=5)*. **B.** Neutralising antibodies to IL-10 receptor were added to cultures alone or in the presence of anti-IL-10 and anti-TGF-β. Bars represent mean suppression of IL-17±SE *(n=3)*.

### 4.6 Patients treated with adalimumab have more Treg expressing CD39 than healthy controls, patients with active RA or patients treated with etanercept.

CD39 is an ectonucleotidase involved in the breakdown of ATP into adenosine, a molecule with known anti-inflammatory properties [433]. In 2009, a paper by Fletcher et al. described a population of $CD39^+$ Treg with the capacity to suppress IL-17 [105]. We examined *ex vivo* expression of CD39 on Treg from RA patients and found that a significantly greater proportion of AdTreg bear the expression of CD39 than HTreg, RATreg or EtTreg (Figure 4.6 A). The breakdown of ATP to AMP and then adenosine requires CD39 to work in tandem with a second enzyme CD73. In mice, it has been shown that CD39 and CD73 co-localise on $FOXP3^+$ Treg [96], though there is some debate about co-expression on human Treg [101-103, 434]. The data here indicate that CD39 and CD73 do co-localise in whole PBMC, however very little CD73 is detected on CD4 cells, despite clear expression of CD39 suggesting that perhaps CD39 and CD73 do not co-localise on human Treg (Figure 4.6. B).

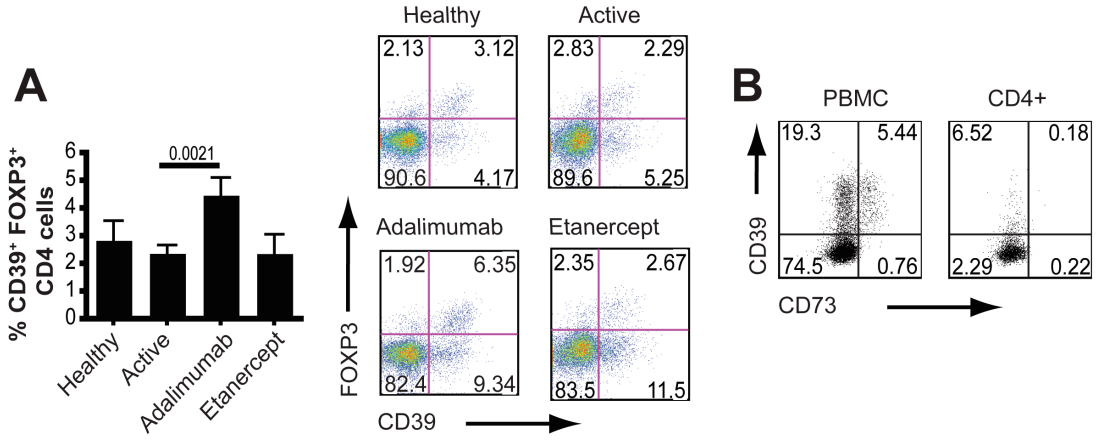


**Figure 4.6. CD39$^+$ Treg are increased in RA patients treated with adalimumab**

**A.** PBMC from healthy controls, patients with active RA and patients treated with adalimumab or etanercept (*n=6-8*) were stained with CD4, CD39 and FOXP3. Graph shows percentage FOXP3$^+$ CD39$^+$ cells. Bars represent the mean±SE. Representative FACS plots shown. **B.** FACS plots showing co-staining of CD39 and CD73 from a healthy individual. The panel on the left is whole PBMC and the panel on the right is gated on CD4 T cells.

## 4.7 CD39 is not required for the suppression of IL-17 by Treg from adalimumab treated RA patients

A second recent paper showed that blockade of CD39 using an anti-CD39 (αCD39) antibody impaired the suppressive function of *in vivo* induced Treg [435]. Using the same blocking antibody, we were able to demonstrate by FACS that it bound effectively to CD39 at 2μg/ml (Figure 4.7 A). In conjunction with a collaborator, Dr. Halima Moncrieffe who had previously published data investigating the function of CD39 [101], we were able to show that the antibody effectively impaired CD39 activity. In this assay, PBMC were supplemented with luciferase conjugated ATP: as the ATP was broken down the amount of light emitted was reduced. In healthy controls, patients with active RA and patients treated with adalimumab, incubation with the blocking antibody resulted in a reduced breakdown of ATP compared to the cells incubated with medium alone. RA PBMC incubated with medium used a substantial amount of ATP in the duration of the assay and so showed the greatest difference when treated with the blocking antibody, thus confirming the effectiveness of the αCD39 antibody as a suppressor of high endonucleotidase activity (Figure 4.7 B).

Finally, we used the αCD39 blocking antibody to determine if CD39 was required for the suppression of IL-17. We incubated AdTreg with either the antibody or with medium for 30 minutes before washing thoroughly and adding to responder T cells and monocytes, as previously described. Under these conditions the ability of Treg to suppress IL-17 was unaffected (Figure 4.7 C). As CD39 is also expressed on other cells, monocytes or responder T cells were treated with αCD39. The data suggest that incubating responder T cells with αCD39 before culturing with untreated

monocytes drove them to produce more IL-17 than a culture of responder T cells and monocytes alone. Similarly, blocking CD39 on monocytes before culturing with untreated responder T cells drove the responder T cell to make up to twice as much IL-17 as a culture of untreated responder T cells and monocytes (Figure 4.7 D), perhaps suggesting that CD39 has a Treg-independent role in the regulation of IL-17 production. However, to discount an effect of the anti-CD39 antibody interacting with monocytes via their Fc receptor expression it would be vital to use an isotype control in any future experiments.

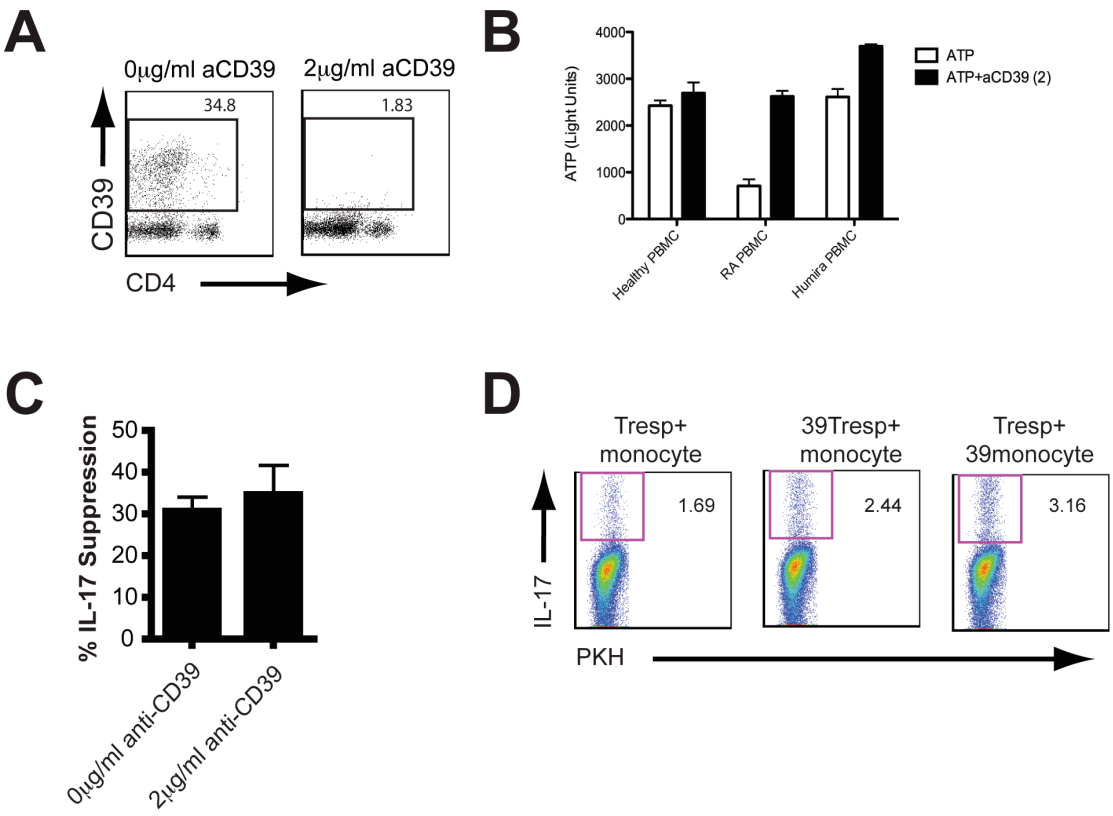


**Figure 4.7. CD39 does not play a role in the suppression of IL-17 by Treg from adalimumab treated patients**

**A.** Whole PBMC from healthy controls were treated for 30 minutes with media (left panel) or 2μg/ml of anti-CD39 (right panel) and then stained for expression of CD4 and CD39. **B** PBMC from healthy controls, patients with active RA and patients treated with humira (the commercial name of adalimumab) (*n=2*) were treated with medium (white bars) or 2μg/ml of anti-CD39 (black bars) and supplemented with an ATP-luciferase construct. After 30 minutes the level of ATP in the well was measured as light emitted. **C.** Treg from adalimumab treated patients were incubated with or without the anti-CD39 antibody for 30 minutes before culturing with autologous responder T cells and monocytes for 5 days. Bars represent mean suppression of IL-17±SE. (*n=5*) **D.** FACS plots of adalimumab treated patients showing IL-17 production from T cells after 5 days. The left panel shows IL-17 production from a culture of Tresponders and monocytes, the middle panel shows IL-17 production when Tresponders were treated with anti-CD39 (39Tresp), before culture with autologous monocytes and the right panel shows IL-17 production from Tresponders cultured with monocytes that had been pre-treated with anti-CD39 (39monocyte). FACS plots are representative of 4 experiments.

## 4.8 Treg pSTAT3 levels in response to TCR stimulation

A paper demonstrating that murine Treg could control Th17 responses in a STAT3 dependent manner [436] led us to investigate whether this signalling molecule was associated with the suppression of IL-17 by AdTreg. Initially, it was investigated if activation of Treg with anti-CD3 and anti-CD28 (αCD3/28) resulted in an increased phosphorylation of STAT3 in adalimumab treated patients compared to patients with active RA or those responding to etanercept. STAT3 phosphorylation was examined using flow cytometry in order to reduce the number of cells required for analysis, but also to remove the need to isolate cell subsets. However, the phosflow antibodies are designed to work in specific buffers, and in particular the STAT antibodies require a buffer with a very high percentage of methanol. This means that any additional surface or intracellular staining that is required must be carefully optimised. We established the optimal conditions to stain CD4 and FOXP3 (Figure 4.8 A), though the quality of FOXP3 staining was clearly impaired by the phosflow buffers. We stimulated PBMC with either a positive control, IL-6, or αCD3/28 then measured pSTAT3 at 15 minutes and 1 hour. 15 minutes is the recommended time required for IL-6 stimulation to phosphorylate STAT3, thus as expected, levels of pSTAT3 increased after 15 minutes stimulation with IL-6. Fifteen minutes stimulation with anti-CD3 and anti-CD28 was not sufficient to activate the STAT3 pathway. After 1 hour of incubation with IL-6 pSTAT3 levels were still elevated compared to unstimulated. Moreover, 1 hour of anti-CD3 and anti-CD28 stimulation was sufficient to phosphorylate STAT3 in both responder T cells and Treg (Figure 4.8 B).

We proceeded to examine STAT3 phosphorylation at 1 hour with anti-CD3 and anti-CD28. Baseline pSTAT3 levels from unstimulated $FOXP3^+$ and $FOXP3^-$ cells were

similar amongst all patient groups but perhaps slightly elevated in healthy controls (Figure 4.8 C/D). After stimulation, the fold induction of pSTAT3 in $FOXP3^+$ and $FOXP3^-$ cells was calculated. In the $FOXP3^-$ population there was a slight increase in pSTAT3 induction in patients with active RA compared to healthy controls or patients treated with adalimumab. Due to a large standard error it is difficult to interpret the data from etanercept treated patients. In $FOXP3^+$ cells there was a trend showing an increased induction of pSTAT3 in AdTreg compared to RATreg ($p=0.09$); there also appeared to be a greater induction of pSTAT3 in AdTreg than in HTreg or EtTreg (Figure 4.8 C/D).

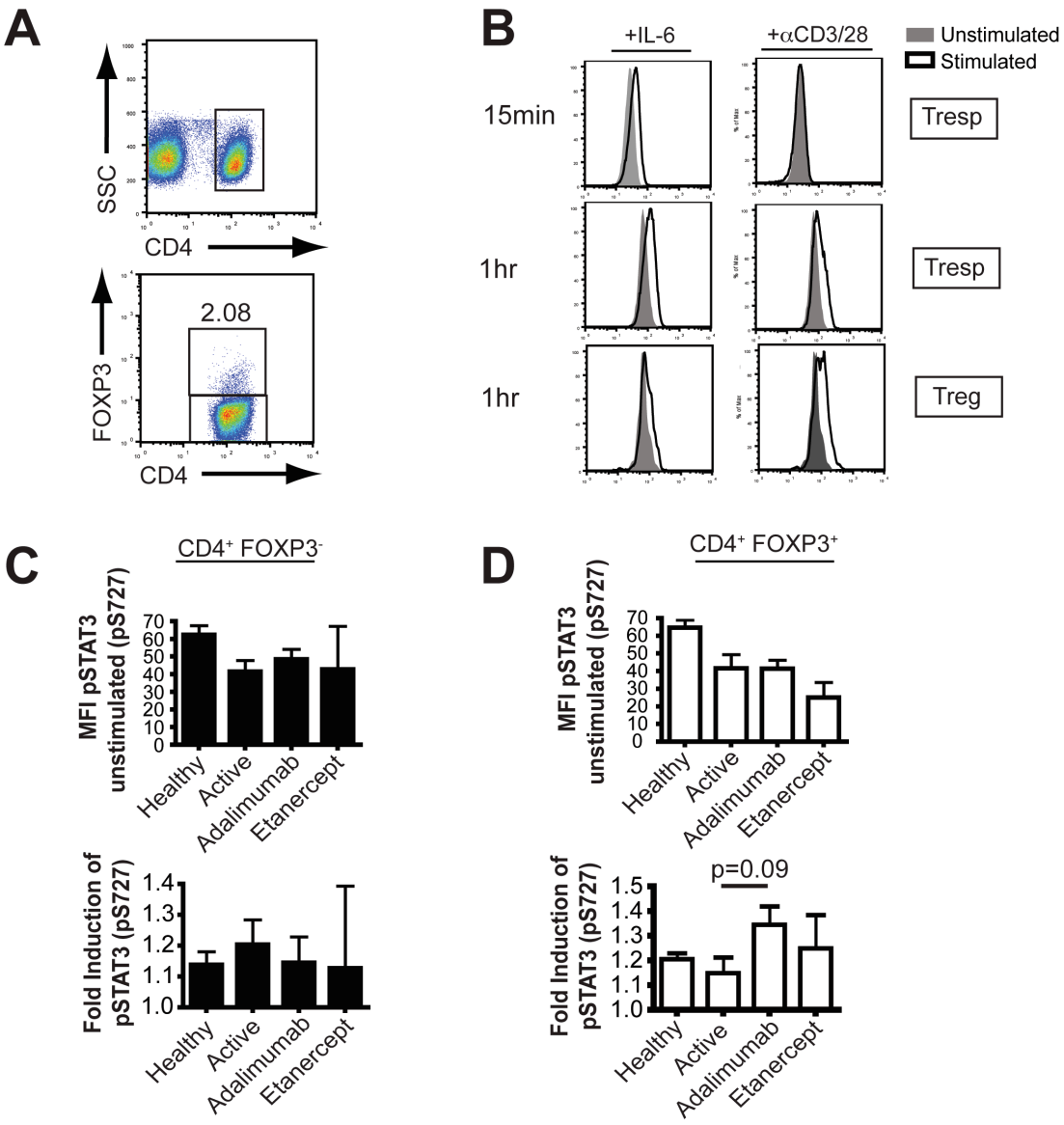


**Figure 4.8. *Ex vivo* STAT3 signalling in T cells from patients with active RA**
**A.** Ex vivo PBMC from a healthy control were stained for CD4 (upper panel) and FOXP3 (lower panel) using BD phosflow buffers. FACS plots are representative of the quality of staining achieved. **B.** Histograms showing pSTAT3. PBMC from an adalimumab treated patient were left unstimulated or treated with αCD3/28 or IL-6 at different time points. The solid grey histograms are unstimulated cells and the outlines are cells that have been stimulated. **C.** pSTAT3 in FOXP3$^-$ cells. The mean fluorescence intensity (MFI) of pSTAT3 was determined in unstimulated cells (upper), this was compared to the MFI of cells stimulated for 1 hour with αCD3/28 and the fold induction of pSTAT3 was calculated (lower). Healthy *n=2*, active *n=7*, adalimumab *n=10*, etanercept *n=5*.Bars represent the mean±SE **D.** pSTAT3 in FOXP3$^+$ cells. As in '**C**' The MFI of pSTAT3 was determined in unstimulated cells (upper) or fold induction of pSTAT3 upon stimulation of αCD3/28 for was calculated (lower). 'n' numbers as in '**C**'. Bars represent the mean±SE.

In the previous experiments, I had been using the STAT3 phospho-serine antibody for determining the phosphorylation state of STAT3 because it had been available in the lab. However, a review of the literature showed that maximal activation of STAT3 requires both tyrosine and serine phosphorylation [437]. Optimisation of these phosflow antibodies showed that they are both activated by IL-6 but the difference from unstimulated was most pronounced with the pTyr705 antibody (Figure 4.9 A). Thus, future experiments utilised pTyr705 phosphorylation as a read out of STAT3 phosphorylation.

In order to validate data shown in Figure 4.8 and to overcome the problems with staining FOXP3 in phosflow buffers, we sorted populations of $CD4^{+}CD25^{+}CD127^{-}$ Treg and $CD4^{+}CD25^{+/-}CD127^{-}$ responder T cells and performed the same experiment, using IL-6 stimulation as a positive control. Due to low 'n' numbers there was substantial variation, but IL-6 stimulation induced pSTAT3 relatively equally in RATreg and AdTreg. There is however, a suggestion that the stimulation of responder T cells with IL-6 induced less phosphorylation of STAT3 in adalimumab treated patients than in patients with active RA (Figure 4.9 B). When stimulated with αCD3/28, responder T cells from patients with active RA had a greater increase in pSTAT3 than responder T cells from patients treated with adalimumab. In contrast, when Treg were stimulated with the αCD3/28 there was a greater induction of pSTAT3 in the AdTreg than RATreg (Figure 4.9 B). Whilst there was a lot of variation and the 'n' numbers were low, these data matched the data gathered *ex vivo*, indicating that pSTAT3 may have some role in the function of AdTreg. Moreover, the similarity between these and the *ex vivo* data suggests that

both pSer727 and pTyr705 antibodies are sufficiently sensitive to detect differences in STAT3 phosphorylation between patient groups.

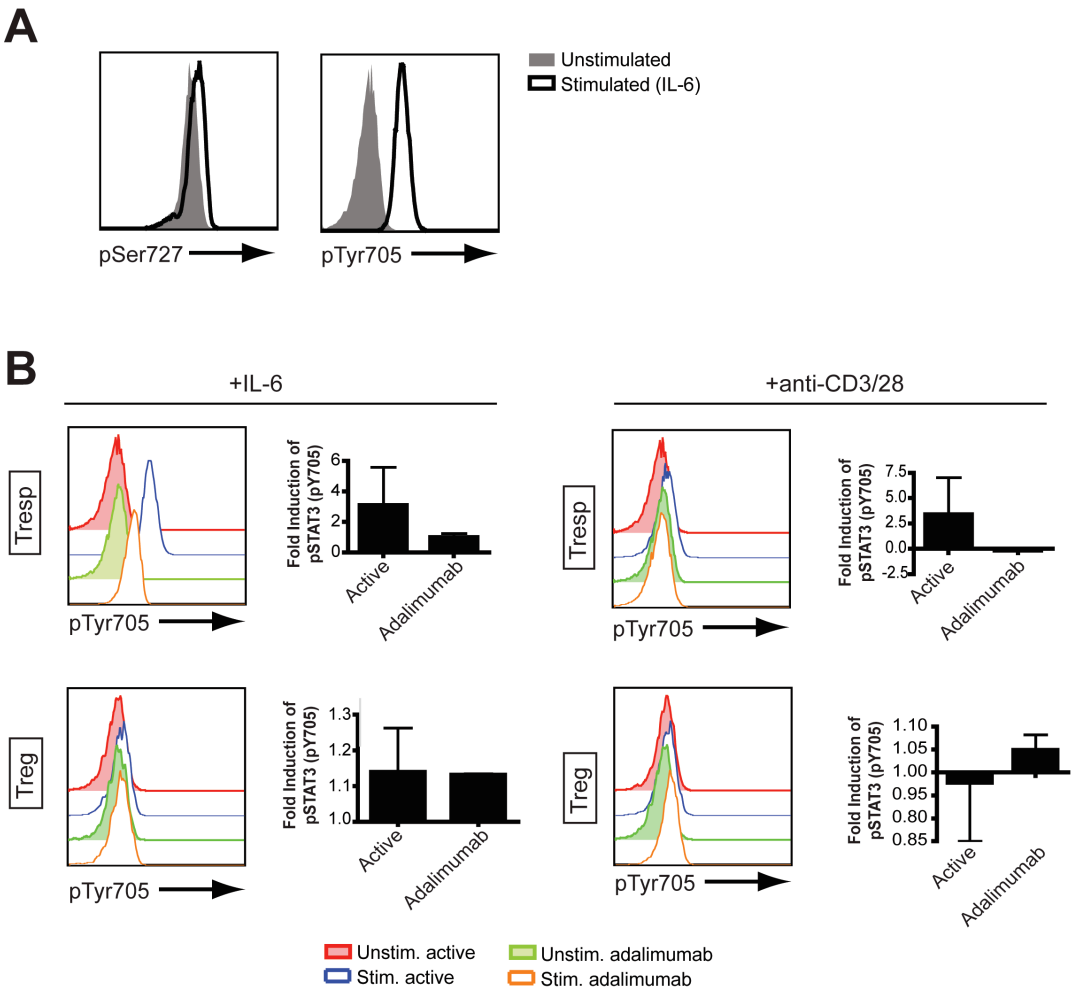


**Figure 4.9. STAT3 signalling in isolated populations of responder T cells and Treg**

**A.** Isolated $CD4^{+}CD25^{-}CD127^{+}$ cells (Tresp) from a patient with active RA were unstimulated or stimulated with IL-6 for 1 hour and then stained for flow cytometry with anti-pSer727 (left panel) or anti-pTyr705 antibodies (right panel). The solid grey histograms are unstimulated cells and the outlines are cells that have been stimulated with IL-6. **B.** Tresp and $CD4^{+}CD25^{+}CD127^{-}$ cells (Treg) were isolated from PBMC. Cells were unstimulated or stimulated with IL-6 or anti-CD3 and anti-CD28 for 1 hour. Histograms depict 1 patient with active RA (solid red are unstimulated and the blue outlines are stimulated cells) and 1 RA patient treated with adalimumab (solid green are unstimulated and the orange outlines are stimulated cells). Graphs show the cumulative fold induction of pSTAT3 (*n=2*). Bars represent the mean fold induction of pSTAT3 ±SE.

## 4.9 Treating cells with a pSTAT3 inhibitor

Next, we purchased an inhibitor specific for STAT3, but not STAT5, an important STAT in Treg differentiation [57]. Using IL-6 to provide a stimulus, we were able to show that the inhibitor abolished induction of pSTAT3 in both responder T cells and Treg (Figure 4.10 A). We then set up an assay to determine if the inhibitor could suppress the function of pSTAT3. Isolated responder T cells were cultured overnight with the inhibitor (*i*Tresp) before adding them to monocytes. After 5 days, cells were re-stimulated and IL-17 production by responder T cells was examined. In comparison to responder T cells and monocytes without inhibitor, it appeared that when *i*Tresp were added to monocytes for 5 days there were fewer cells at the end of culture (Figure 4.10 B). Moreover, when we examined the T cells by FACS, *i*Tresp did not produce IL-17, as might be expected, but they also failed to produce IFNγ. This suggested that the activity of the inhibitor or the DMSO in which it was resuspended was killing the cells. As a result, it was decided to titrate the antibody so as to use the lowest possible inhibitory concentration in future experiments. This experiment had only an 'n' of one, but it seemed as though all concentrations of the inhibitor tested reduced the level of phosphorylation to that of unstimulated cells or lower (Figure 4.10 D). That the level of phosphorylation dropped below that of the unstimulated in some conditions may suggest that there is some background phosphorylation of STAT3 in unstimulated cells. In future experiments an inhibitor concentration of 0.039μM should be sufficient to inhibit STAT3 phosphorylation however, this could be titrated further and the viability of responder T cells and Treg after culture needs to be tested. Once the inhibitor is optimised, inhibitor treated Treg can be added to cultures of responder T cells and monocytes to determine the role of STAT3 in the suppression of IL-17.

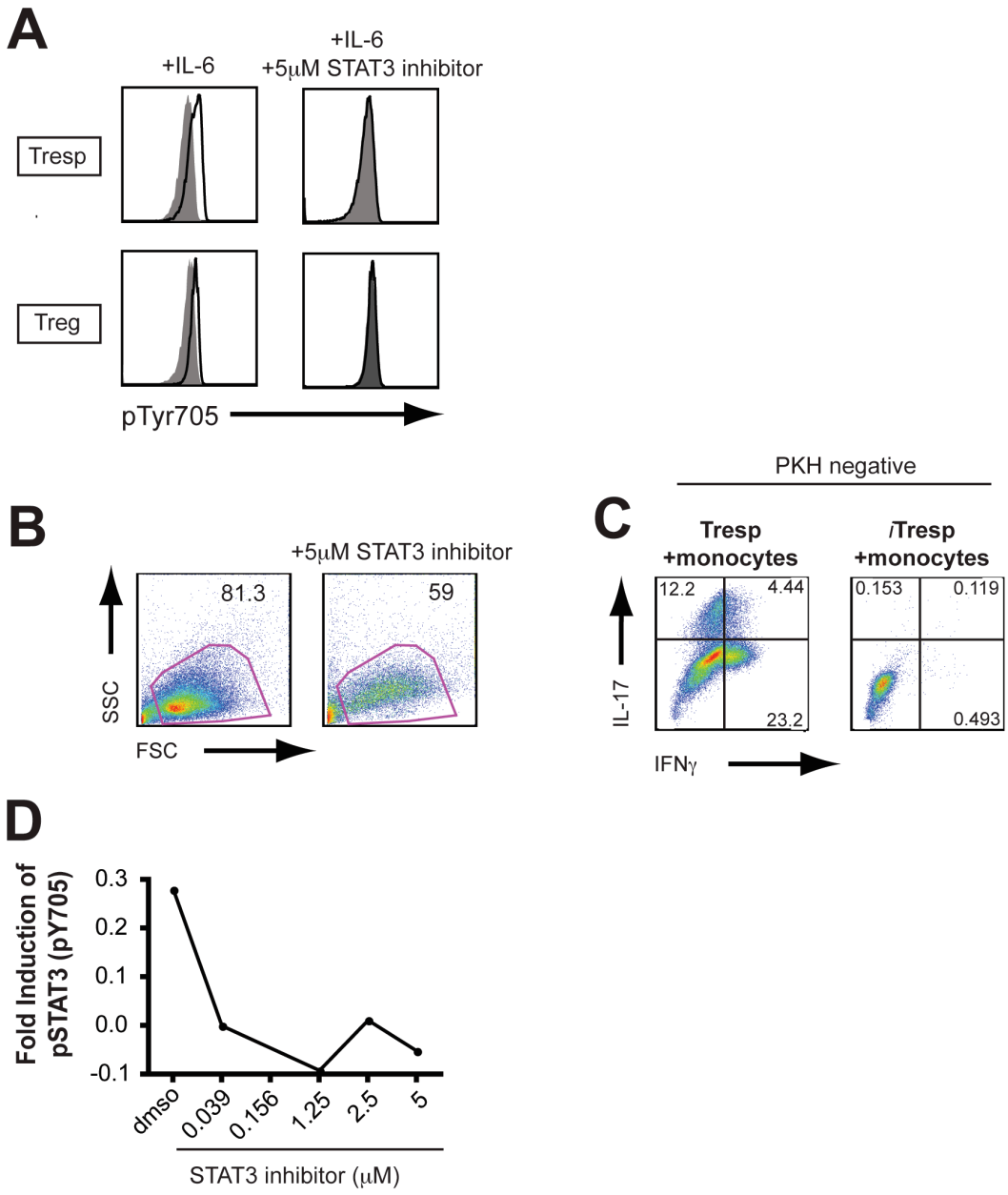


**Figure 4.10. Inhibition of pSTAT3**
**A.** Responder T cells (Tresp) and Treg from adalimumab treated patients were stimulated with IL-6 for 15 minutes in the presence or absence of 5μM of pSTAT3 inhibitor (*n=1*). **B.** Live cell gate after 5 days culture of isolated Tresp and $CD14^+$ monocytes. Tresp were untreated (left panel) or treated overnight with the inhibitor before addition to monocytes for 5 days (right panel). **C.** IL-17 and IFNγ production from T cells after 5 days culture. FACS plots show cultures of Tresp and monocytes, inhibitor treated Tresp (iTresp) and monocytes. **D.** Graph shows the fold induction of pSTAT3 in cells treated with STAT3 inhibitor. Whole PBMC from an RA patient treated with adalimumab were treated with 0.02% DMSO or with indicated concentrations of inhibitor (diluted in 0.02% DMSO) for 45 minutes. Cells were then supplemented with media (unstimulated) or stimulated with IL-6 for 15 minutes before staining with anti-CD4 and anti-pTyr705 antibodies. The fold induction of pTyr705 in CD4 cells stimulated with IL-6 was determined for each concentration of inhibitor (*n=1*).

### 4.10 Neutralisation of the p35 subunit of IL-35

It has been suggested that IL-35 production from Treg can control Th17 responses [91], though very little is known about the role of IL-35 in human Treg. A recent paper described the use of a neutralising antibody targeted at the p35 chain of IL-35 which specifically inhibited IL-35, but not other members of the IL-12 family, including IL-12, IL-27 and IL-23 [438]. We used the same antibody to elucidate a possible role for IL-35 in the ability of AdTreg to suppress IL-17. Low 'n' numbers resulted in large variability so there were no significant differences and the experiment would need to be repeated generate sufficient power from which to make conclusions (Figure 4.11).

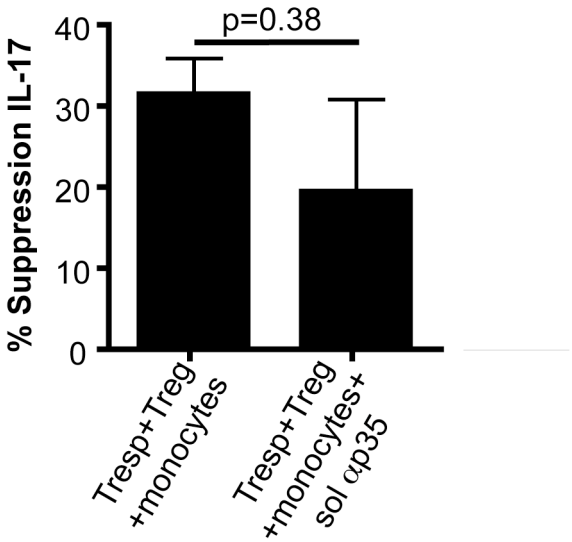


**Figure 4.11. Blocking IL-35 p35 subunit**

Treg, responder T cells and monocytes from adalimumab treated patients were cultured for 5 days in the presence or absence of anti-p35 antibody (*n=3*). Bars represent the mean suppression of IL-17 ±SE.

## 4.11 Suppression of IL-17 by adalimumab Treg is mediated through the suppression of IL-6

We had originally supplemented the co-culture assay with monocytes because monocyte-derived cytokines such as IL-6, IL-1β and IL-23 [194, 439, 440] are known to be important drivers of Th17 cells. Having investigated a direct pathway of Treg suppression of IL-17, we considered the possibility that AdTreg may be suppressing Th17 cells in an indirect manner, via the suppression of monocytes. Thus, the production of IL-23 was measured in cultures containing monocytes and stimulated responder T cells from adalimumab treated patients with or without Treg. Very little IL-23 was detected, but it was clear that addition of Treg to responder T cells and monocytes did not reduce IL-23 production (Figure 4.12 A). IL-6 and IL-1β are both known to be early inflammatory mediators, however IL-1β production from all of the patient groups studied was very low (Figure 4.12 B) and neither AdTreg nor EtTreg showed a capacity to suppress it ($p=0.1$) (Figure 4.12 C). Interestingly, levels of IL-6 were the highest in cultures of responder T cells and monocytes from patients with active RA and those treated with adalimumab and lower in etanercept treated patients (Figure 4.12 B). Nevertheless, IL-6 production was reduced upon the addition of autologous Treg to adalimumab T responders and monocytes. This was not observed in patients with active RA or those treated with etanercept, but rather, IL-6 increased following addition of these Treg, paralleling their ability to increase rather than suppress IL-17 production (Figure 4.12 D).

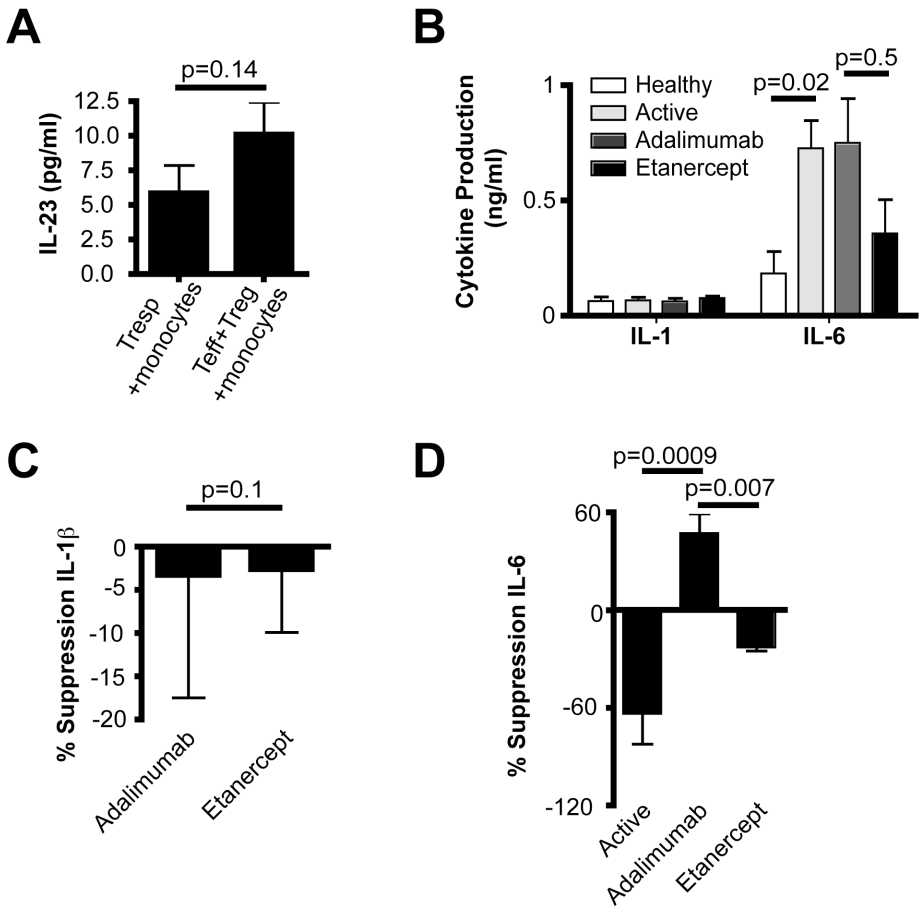


**Figure 4.12. Treg from patients treated with adalimumab suppress IL-6, but not IL-23 or IL-1β**

**A.** Responder T cells and monocytes from adalimumab treated patients were cultured with and without Treg. After 5 days supernatants were assayed for IL-23 by ELISA (*n=6*). Bars represent mean IL-23 ±SE. **B.** Supernatants from cultures of responder T cells and monocytes from healthy controls and the different patient groups were assayed for IL-1β and IL-6 by CBA (*n=5-10*). **C**. Suppression assays were performed with samples from adalimumab and etanercept treated patients and IL-1β concentration was determined by CBA. Suppression was calculated as described in the methods (adalimumab *n=8*, etanercept *n=5*). Bars represent the mean suppression of IL-1β ±SE. **D.** IL-6 concentration from supernatants of suppression assays was determined by CBA and suppression of IL-6 was calculated (active *n=4*, adalimumab *n=8*, etanercept *n=5*). Bars represent the mean suppression of IL-6 ±SE.

In order to confirm a link between IL-6 and the suppression of IL-17 in these assays, an IL-6 neutralising antibody was added to cultures of adalimumab monocytes and responder T cells. This reduced production of IL-17 from responder T cells, imitating the effects of adding AdTreg to the culture (Figure 4.13 A). As a control, an IL-1β neutralising antibody was also used in these assays. Surprisingly, the effect of blocking IL-1β was identical to blocking IL-6 (Figure 4.13 A). Analysis of the culture supernatants showed that IL-6 production was abolished by anti–IL-6 but was also dramatically reduced by anti-IL-1β (Figure 4.13 B). In contrast, whilst anti–IL-1βblocked IL-1β production and this was not altered by the combined blockade of IL-1β and IL-6, the blockade of IL-6 alone had no impact on the production of IL-1β (Figure 4.13 C). This suggests that IL-6 is the key mediator of IL-17 production by T cells in RA patients treated with adalimumab. Indeed, the suppression of IL-17 by AdTreg was reversed by the addition of 20ng/ml recombinant human IL-6 to cultures of responder T cells, monocytes and Treg from adalimumab treated patients (Figure 4.13 D).

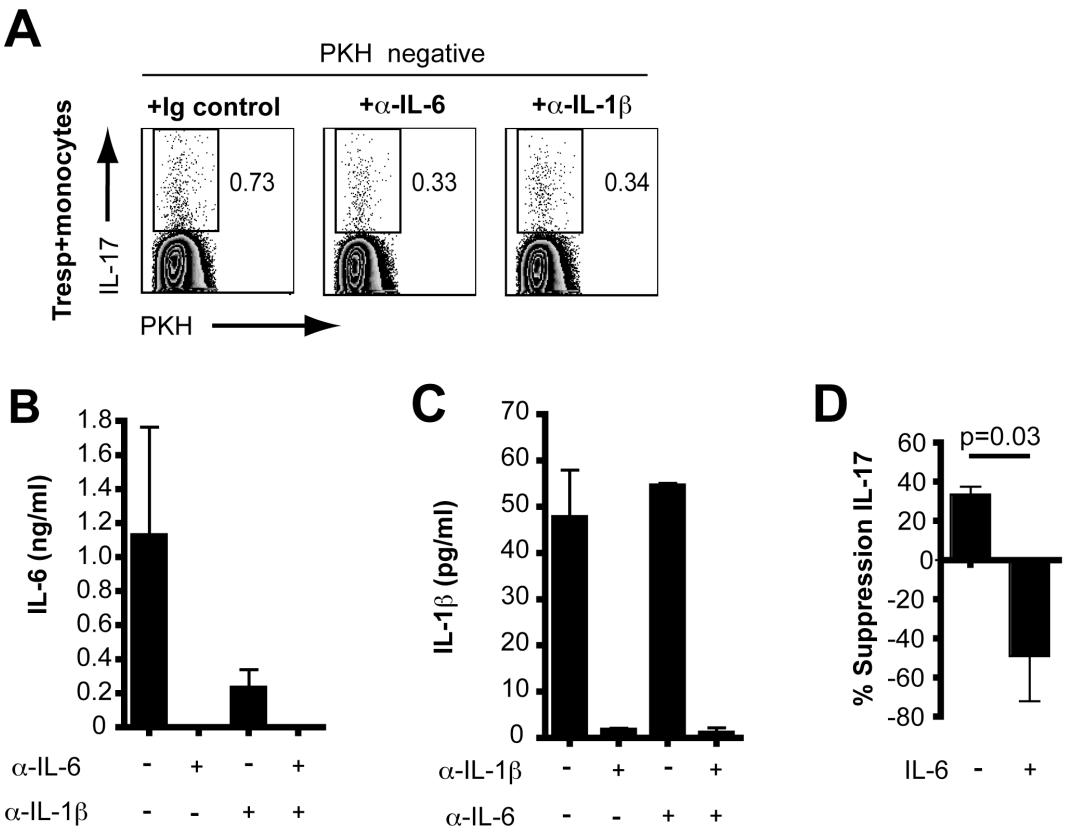


**Figure 4.13. IL-17 suppression by AdTreg is reversed by the addition of IL-6**

**A.** IL-17 production from T cells after culture for 5 days. Responder T cells and monocytes were cultured with an isotype control antibody (left panel), an anti-IL-6 antibody (middle panel) or an anti-IL-1β antibody (right panel). FACS plots are representative of 4 independent experiments. **B.** IL-6 production from responder T cells and monocytes as determined by CBA after 5 days of culture with an isotype control antibody (-), an anti-IL-6 neutralising antibody or an anti-IL-1β neutralising antibody as indicated (*n=4*) **C.** IL-1β production from responder T cells and monocytes as determined by CBA after 5 days of culture with an isotype control antibody (-), an anti-IL-1β neutralising antibody or an anti-IL-6 neutralising antibody as indicated (*n=4*) **D.** Suppression of IL-17 by AdTreg cultured in the presence (+) or absence (-) of IL-6 (20ng/ml) (*n=4*).

### 4.12 The suppression of IL-17 and IL-6 by AdTreg does not require cell-cell contact

Data from other groups has suggested that Treg suppression of monocytes is at least partly contact dependent [441]. In order to determine if the ability of AdTreg to suppress monocyte-driven IL-17 was contact dependent, the co-culture experiments described above were repeated in a transwell plate. AdTreg were cultured in contact with responder T cells and monocytes in the lower chamber of a transwell plate, or plated in the upper chamber where they were separated from responder T cells and monocytes by a permeable membrane. Suppression of IL-17 was unaffected when contact was prevented between AdTreg and their autologous responder T cells and monocytes (Figure 4.14 A). This was confirmed by CBA analysis of culture supernatants (Figure 4.14 B). Furthermore, separation of Treg from responder T cells and monocytes did not impair IL-6 suppression (Figure 4.14 C). Thus, the capacity of AdTreg to suppress Th17 responses is mediated by a soluble factor.

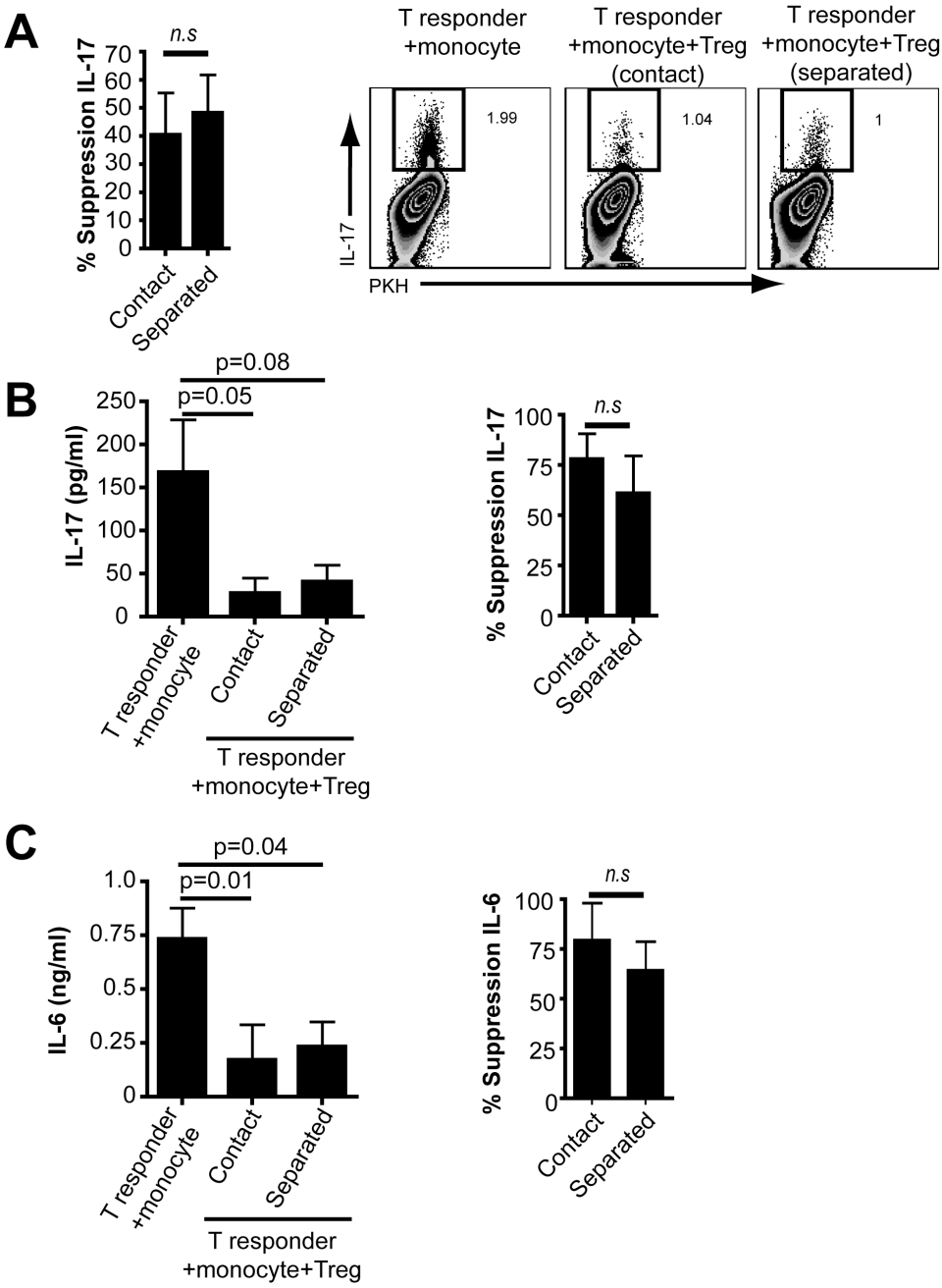


**Figure 4.14. Suppression of IL-17 is not contact dependent**

**A.** Treg from patients responding to adalimumab were cultured in a transwell plate either in contact with autologous monocytes (contact) and responder T cells, or separated by a permeable membrane (separated). Bars represent suppression of IL-17 ±SE as measured by FACS ($n=5$). Representative FACS plots are shown. **B.** Supernatants from ***A*** were assayed for IL-17 by CBA. The left-hand graph shows absolute levels of IL-17 ±SE. The right-hand graph shows the calculated suppression of IL-17 ±SE. **C.** Supernatants from ***A*** were assayed for IL-6 by CBA. The left-hand graph shows absolute levels of IL-6 ±SE. The right-hand graph shows the calculated suppression of IL-6 ±SE. (n.s.) - not significant.

Summary:

As previously published, this chapter has shown that RATreg are defective in their ability to suppress IFNγ. Suppression of this cytokine is restored to healthy levels in patients responding to adalimumab, but not patients responding to etanercept. Depletion of Treg from whole PBMC showed that neither HTreg nor RATreg demonstrated the capacity to regulate IL-17 production. In contrast, AdTreg could regulate IL-17 production from whole PBMC but his was not seen in patients responding to etanercept. Co-culture experiments showed that the presence of monocytes could drive IL-17 production from T cells and suppression assays where Treg were added to cultures of responder T cells and monocytes confirmed that AdTreg, but not HTreg, RATreg or EtTreg, could suppress IL-17. In contrast to the suppression of IFNγ, suppression of IL-17 by AdTreg was not mediated via IL-10 and TGFβ.

Further investigations showed that the activity of CD39 on AdTreg was not responsible for the capacity of these cells to suppress IL-17. Examination of pSTAT3 expression in $FOXP3^{+}$ cells showed that AdTreg express elevated levels of pSTAT3 upon TCR stimulation and this may implicate STAT3 in the capacity of these cells to suppress IL-17. Finally, it was shown that AdTreg regulate Th17 cells via the modulation of monocyte-derived IL-6 in a contact independent manner.

# Chapter 5

## Immunological changes in rheumatoid arthritis patients treated with tocilizumab

Objectives:

1. Utilise flow cytometry to determine the levels of regulatory T cells and Th17 cells in RA patients before and after tocilizumab therapy.

## 5.1 Treg and Th17 cell levels are unchanged in patients treated with tocilizumab

The data in the previous chapter highlighted the importance of IL-6 in IL-17 mediated inflammation. Thus, I proceeded to study a small cohort of RA patients due to commence a novel anti-IL-6 receptor therapy, tocilizumab. These patients all had active RA and had failed conventional DMARD therapy as well as anti-TNF and, in some cases, rituximab. The data presented in this chapter comes from a cohort of 8 patients. A substantial portion of the data is cross-sectional, though for some aspects of the study I had access to PBMC from the same patients before and after therapy.

Peripheral blood samples were collected from patients immediately prior to their first infusion and at 6 weeks post therapy, prior to their second infusion. There was a lot of variation between patients studied, although - when compared to healthy controls – there was a trend for the percentage of $FOXP3^+$ cells to be much higher in patients before therapy with tocilizumab ($p=0.09$). Furthermore, the patients studied appeared to divide into 2 groups. Further investigation revealed that those patients with the highest levels of FOXP3 expression were rheumatoid factor (RF) negative whilst the patients with lowest expression of FOXP3 were RF positive. This may be indicative of different therapeutic histories, as RF positive patients are more likely to have previously been treated with the B cell depleting agent, rituximab. Irrespective of RF positivity, tocilizumab therapy had no effect on peripheral Treg numbers in the patients studied (Figure 5.1 A).

When $RORC^+$ Th17 cells were examined, compared to healthy controls percentage Th17 cells were elevated in RA patients before tocilizumab therapy ($p=0.009$).

However, after therapy the levels of $RORC^+$ cells were unchanged. Next, the population of Treg that also express RORC were examined: to explore whether this was altered after therapy with tocilizumab. Whilst levels of these double positive cells were relatively low overall, RA patients before tocilizumab therapy had raised levels compared to healthy controls ($p=0.04$). After therapy, levels of these cells were variable but there was no significant difference from patients before therapy ($p=0.34$)(Figure 5.1 C).

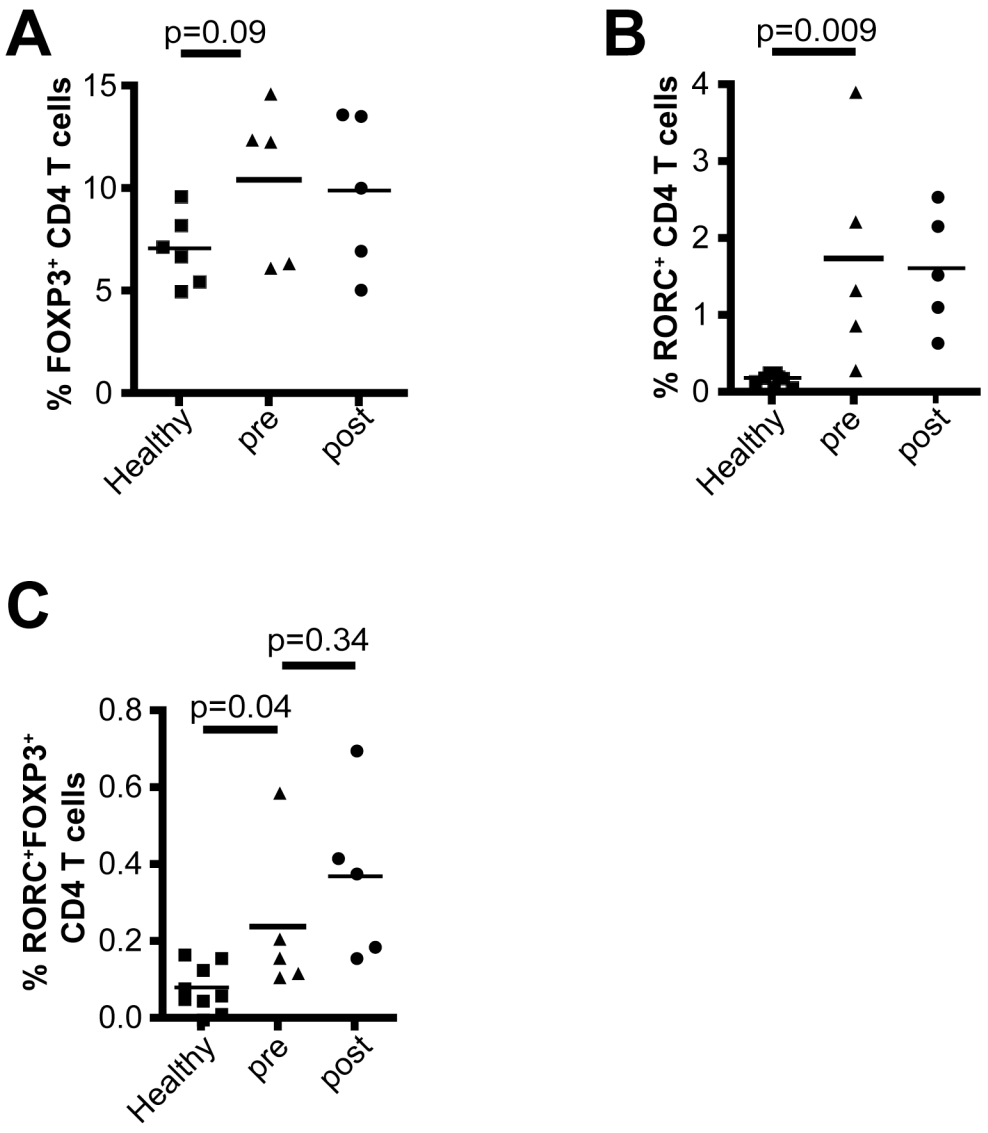


**Figure 5.1. Treg and Th17 cells in RA patients treated with tocilizumab.**

*Ex vivo* PBMC from patients before (pre) and after (post) therapy with tocilizumab were stained for flow cytometry with CD4, FOXP3 and RORC. **A.** Percentage $CD4^+FOXP3^+$cells. **B.** Percentage $RORC^+$ CD4 T cells **C.** Percentage $FOXP3^+RORC^+$ CD4 T cells. For all graphs points show individual donors and bars show mean percentage.

## 5.2 CD4$^+$ T cells after tocilizumab therapy

In addition to examining the Th17/Treg balance in these patients, other features of T cells that might be altered by the neutralisation of IL-6 signalling were examined. The percentage of CD4$^+$ T cells was the same in patients before and after therapy (Figure 5.2 A). This was mirrored by data showing that T cell proliferation *ex vivo* was similar before and after therapy, as determined by Ki67 staining (Figure 5.2 B).

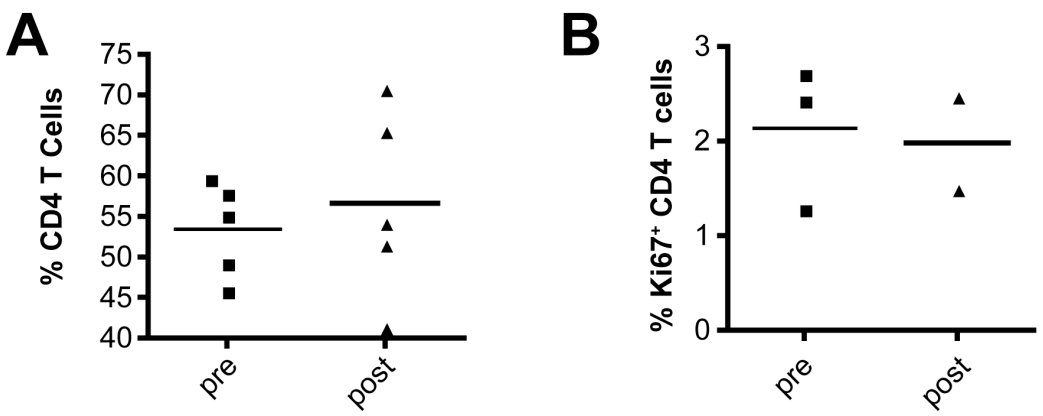


**Figure 5.2. CD4 T cell proliferation after tocilizumab therapy.**

**A**. Percentage CD4 T cells from patients before (pre) and after (post) therapy with tocilizumab. **B.** Percentage Ki67$^+$ CD4 T cells before (pre) and after (post) therapy. Points show individual donors and bars show mean percentage.

Samples from the same patient before and after therapy were used to determine the effects of tocilizumab on T cell cytokine production. Levels of TNF were variable between individuals and were differentially affected by tocilizumab, making it difficult to determine the effect of neutralising IL-6 on TNF production (Figure 5.3A). Surprisingly, in 2 of 3 matched pairs, the neutralisation of IL-6 receptor appeared to drive the production of IL-17, though this increase was not significant ($p=0.71$) (Figure 5.3 B). Similarly, the changes in IL-10 production after therapy were not consistent, with 2 patients showing very small changes and a third patient showing a large increase in IL-10 (Figure 5.3 C). Thus, despite being matched pairs there is too much variability in individual response to therapy to make conclusions about the effect of IL-6 blockade on cytokine production by T cells.

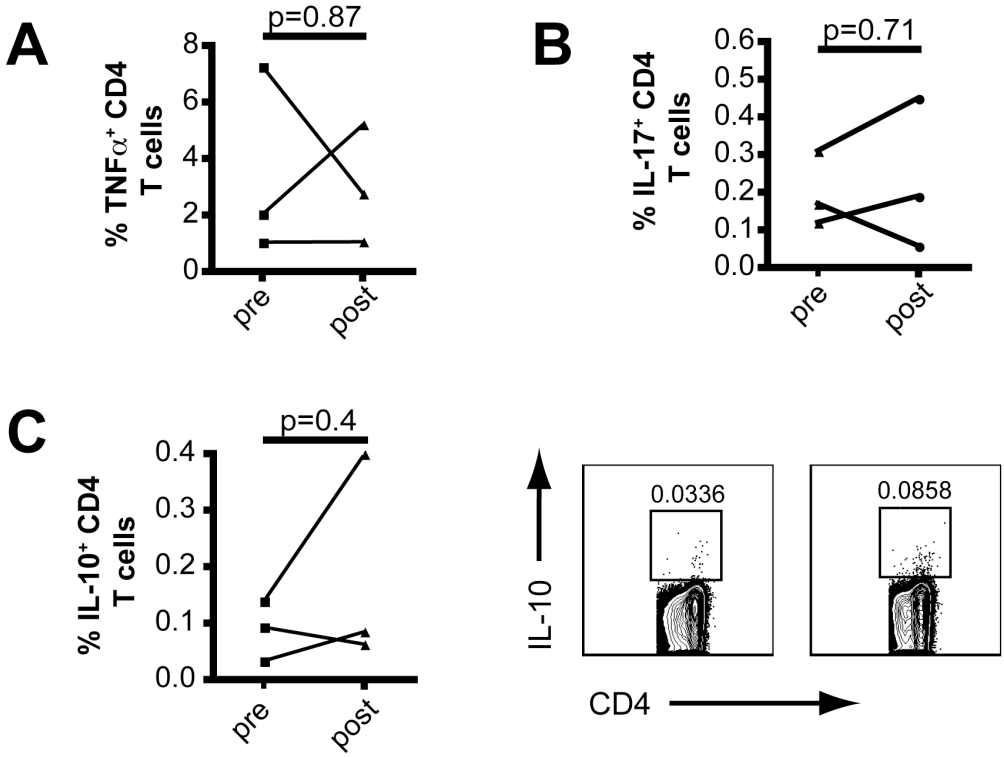


**Figure 5.3. T cell cytokine production after tocilizumab therapy.**

PBMC from matched patients before (pre) and after (post) therapy were thawed and stained for CD4 before stimulation with PMA for 4 hours. **A.** Percentage $TNF^+$ T cells. **B.** Percentage of IL-$17^+$ T cells. **C.** Percentage IL-$10^+$ T cells. Representative FACS plots show a matched patient sample 'pre' and 'post' therapy. On all graphs connected dots represent the same patient before and after therapy.

## 5.3 Changes in B cells after tocilizumab therapy

As IL-6 is an important B cell growth factor, the B cell population was examined for changes after tocilizumab therapy. There was a trend for an increased percentage of CD19 B cells overall (p=0.09) (Figure 5.4 A). This was not explained by an increased proliferation of B cells as the percentage of $Ki67^+$ B cells was unaffected after therapy (p=0.36) (Figure 5.4 B). Levels of immature B cells, which have been described as regulatory, appeared to fall slightly after therapy, though this was not significant (p=0.36) (Figure 5.4 C). Similarly, there were no differences in proliferation of immature B cells (Figure 5.4 D). Production of both pro-inflammatory (TNF) (Figure 5.4 E) and anti-inflammatory (IL-10) (Figure 5.4 F) cytokines by B cells was variable and there were no consistent changes after therapy.

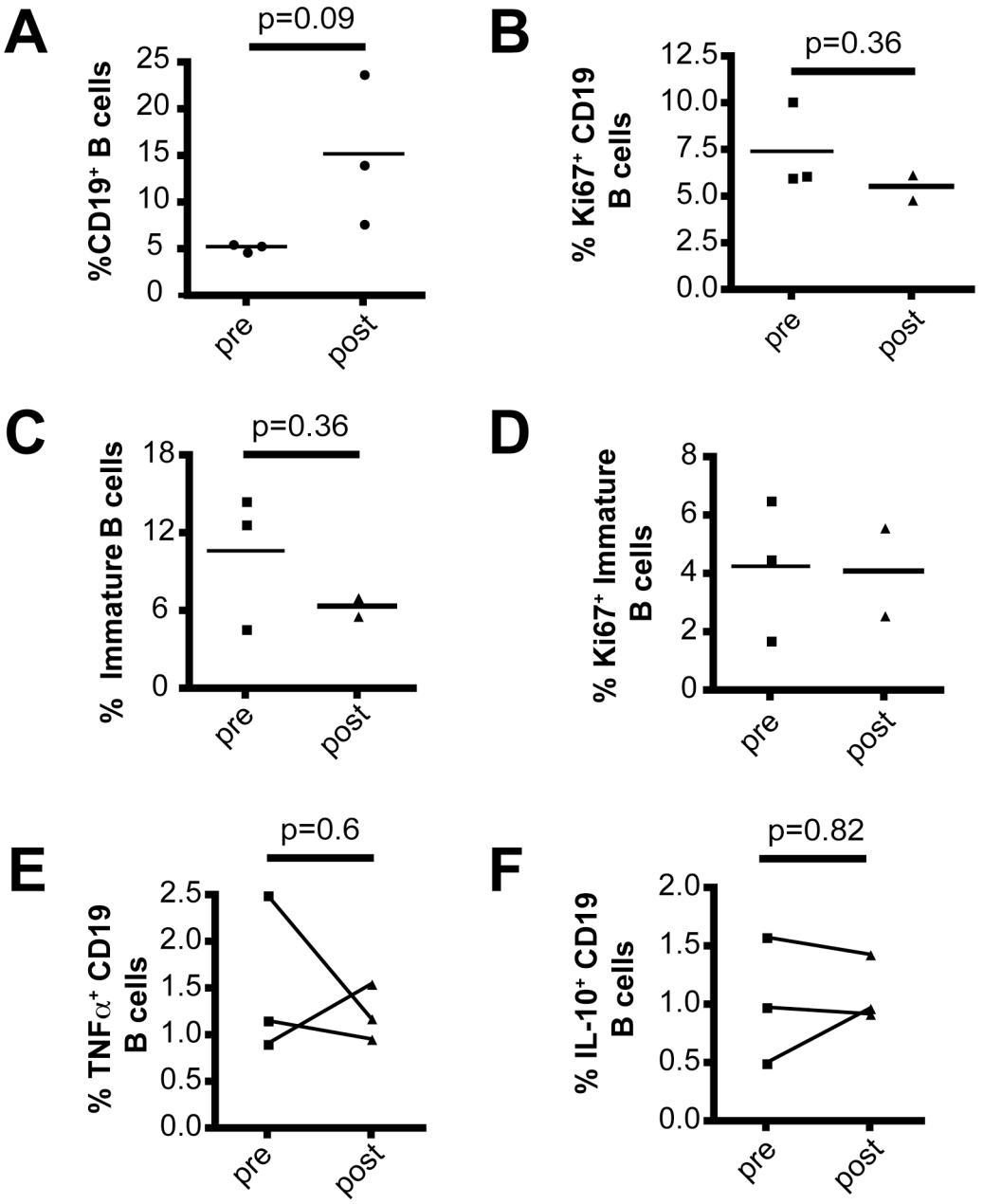


**Figure 5.4. Changes in B cell populations after tocilizumab therapy.**

**A.** Percentage $CD19^+$ cells from patients before (pre) and after (post) therapy with tocilizumab. Points show individual donors and bars show mean percentage. **B.** Percentage $Ki67^+$ B cells from patients before and after therapy with tocilizumab was determined. **C.** Percentage immature B cells ($CD19^+CD24^{hi}CD38^{hi}$) from patients before and after therapy with tocilizumab. **D.** Percentage $Ki67^+$ immature B cells from patients before and after therapy with tocilizumab. **E.** Percentage $TNF\alpha^+$ $CD19^+$ B cells from 3 matched patients. Connected dots represent the same patient before and after therapy. **F.** Percentage IL-$10^+$ $CD19^+$ B cells from 3 matched patients.

## 5.4 A reduction in memory B cells and IL-21 production after therapy with tocilizumab

It has been reported that tocilizumab therapy resulted in a fall in the percentage of memory B cells [384]. When the levels of $CD27^{+}IgD^{-}$ memory B cells were investigated in patients treated with tocilizumab, there was a decrease in this population after therapy in 3 patients (Figure 5.5 A). Furthermore, a drop in the percentage of antibody producing plasma cells was observed after tocilizumab therapy (Figure 5.5 A). Interestingly, this drop in the percentage of memory and plasma B cells was matched by a fall in IL-21 production by T cells in these patients (Figure 5.5 B). Indeed, IL-21 has been shown to be critical for the development of memory B cells [442] so these two observations may be linked. However, this requires further study of sufficient power to determine if these differences represent a consistent outcome of therapy.

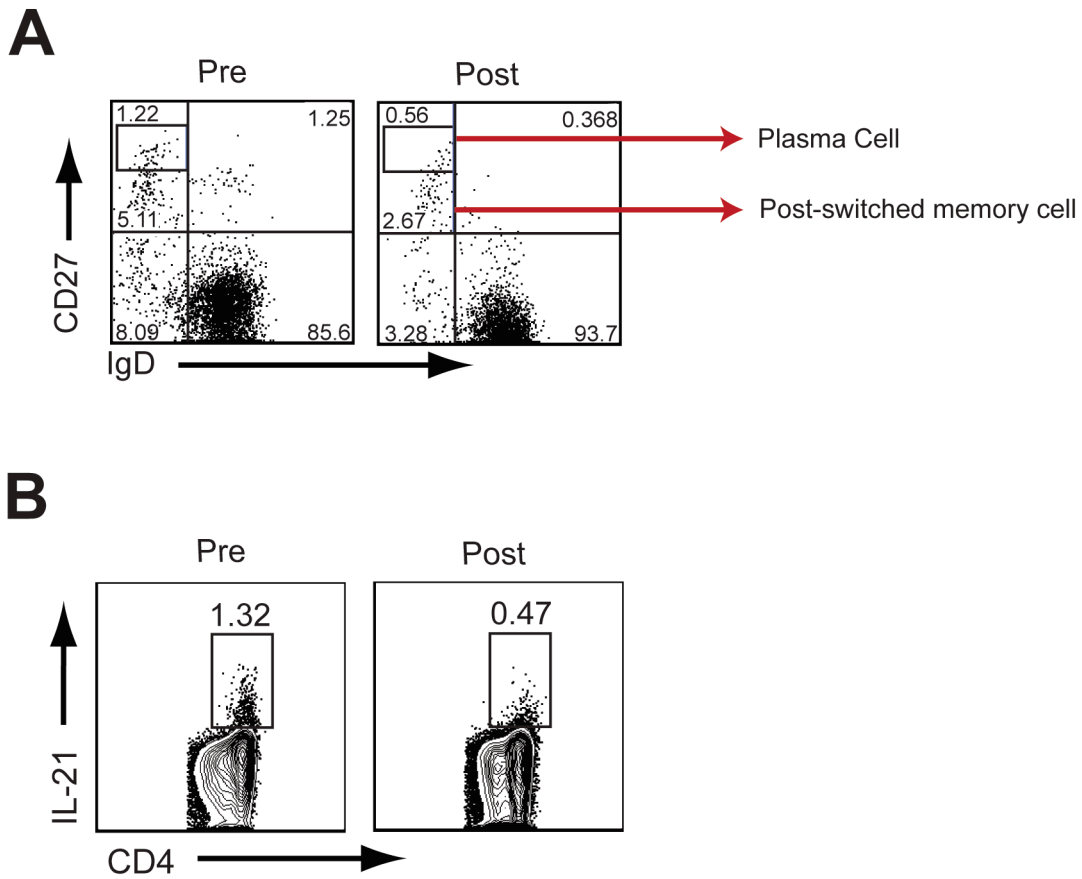


**Figure 5.5. Memory B cell population, plasma cells and IL-21 production by T cells is reduced after tocilizumab.**

**A.** Whole PBMC were stained ex-vivo for B cell markers. FACS plots show staining of CD27 and IgD on the $CD19^+$ B cell population in a matched patient sample 'pre' and 'post' therapy. Plasma cells are identified at $IgD^-CD27^{hi}$ and post-switched memory cells are identified as $IgD^-CD27^+$Representative of 3 matched pairs. **B.** FACS plots show Percentage $IL\text{-}21^+$ CD4 T cells in a matched patient sample 'pre' and 'post' therapy. Representative of 2 matched pairs.

## 5.5 Changes in STAT3 signalling after tocilizumab therapy

Finally the phosphorylation of STAT3 was examined, a key downstream factor in IL-6 signalling. There were no consistent differences in the baseline level of pSTAT3 in patients before or after tocilizumab therapy (Figure 5.6 A). In contrast, upon stimulation with IL-6 all patients showed an increase in pSTAT3. However, in patients treated with tocilizumab, the level of pSTAT3 induced was much lower than untreated patients (Figure 5.6B). Due to the variability in the baseline levels of pSTAT3, this reduction in pSTAT3 upon stimulation was not reflected when fold induction of pSTAT3 was calculated (Figure 5.6 C).

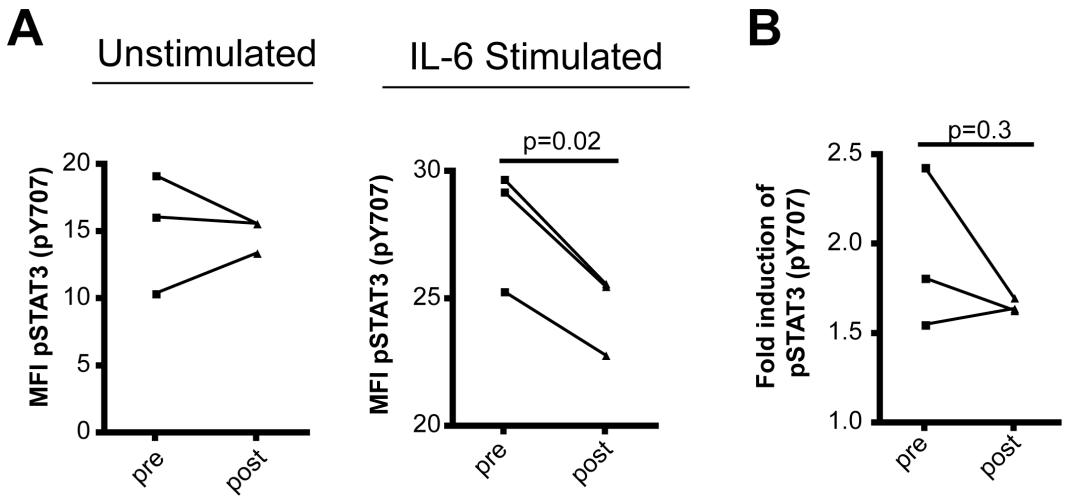


**Figure 5.6. STAT3 signalling in patients treated with tocilizumab.**

Ex vivo PBMC from patients before (pre) and after (post) therapy with tocilizumab were left unstimulated or stimulated with IL-6 for 15 minutes before staining with CD4 and pSTAT3 antibodies for flow cytometry **A**. The mean fluorescence intensity (MFI) of pSTAT3 was determined in unstimulated cells and cells stimulated with IL-6, **B.** Fold induction of pSTAT3 by IL-6 was calculated. Connected dots represent the same patient before and after therapy.

Summary:

Compared to patients who had yet to commence tocilizumab treatment, 6 weeks after therapy there were no differences in the percentage of Th17 cells *ex vivo*. Additional investigation saw no differences in Treg levels or production of IL-10 and TNF from $CD4^+$ T cells or $CD19^+$ B cells. In contrast, a fall in plasma B cells was associated with a reduction in IL-21 production from T cells after therapy with tocilizumab. pSTAT3 expression in response to IL-6 stimulation was lower in patients after therapy with anti-IL-6 receptor, though when the fold induction of pSTAT3 was calculated the differences before and after therapy failed to reach significance.

# Chapter 6

## Immunological changes in psoriatic arthritis patients treated with anti-TNF

Objectives:

1. Determine the percentage of Treg in patients with active PsA and compare to patients with PsA responding to adalimumab or etanercept.
2. Utilise a Treg depletion approach to explore the capacity of Treg from active PsA patients and anti-TNF treated PsA patients to suppress IL-17 and IL-22.

## 6.1 The effects of adalimumab treatment on regulatory T cell numbers is specific to patients with RA

To investigate if the increase in Treg in the peripheral blood of RA patients treated with adalimumab is disease specific, the percentage of Treg from patients with active psoriatic arthritis (PsA) and PsA patients treated with anti-TNF was determined. There was no difference in the percentage of Treg between healthy controls, patients with active PsA and patients treated with anti-TNF, suggesting that the increase in Treg following adalimumab therapy is specific for RA (Figure 6.1A).

## 6.2 Th17 cells are reduced in PsA patients treated with anti-TNF

In accordance with published data showing that Th17 cells are increased in the periphery of PsA patients [443], $RORC^+$ CD4 cells were also significantly increased in patients with active PsA compared to healthy controls (p=0.009). There was a trend towards a reduction in $RORC^+$ cells in adalimumab treated patients (p=0.08) but whilst the percentage of $RORC^+$ cells in etanercept treated patients also appeared to fall this failed to reach significance (p=0.28) (Figure 6.1 B). In contrast, upon examination of IL-17 production by these cells there was a trend towards a fall in IL-$17^+$ T cells after treatment with etanercept (p=0.07) but not adalimumab (p=0.2) (Figure 6.1 C). Increasing the 'n' number of these studies should provide sufficient power to confirm a drop in Th17 cells in PsA patients treated with anti-TNF. The *ex vivo* production of IFNγ was similar in healthy controls, patients with active PsA and patients treated with adalimumab. In contrast, there was a trend for production of IFNγ to be reduced in patients treated with etanercept (p=0.08) (Figure 6.1 D).

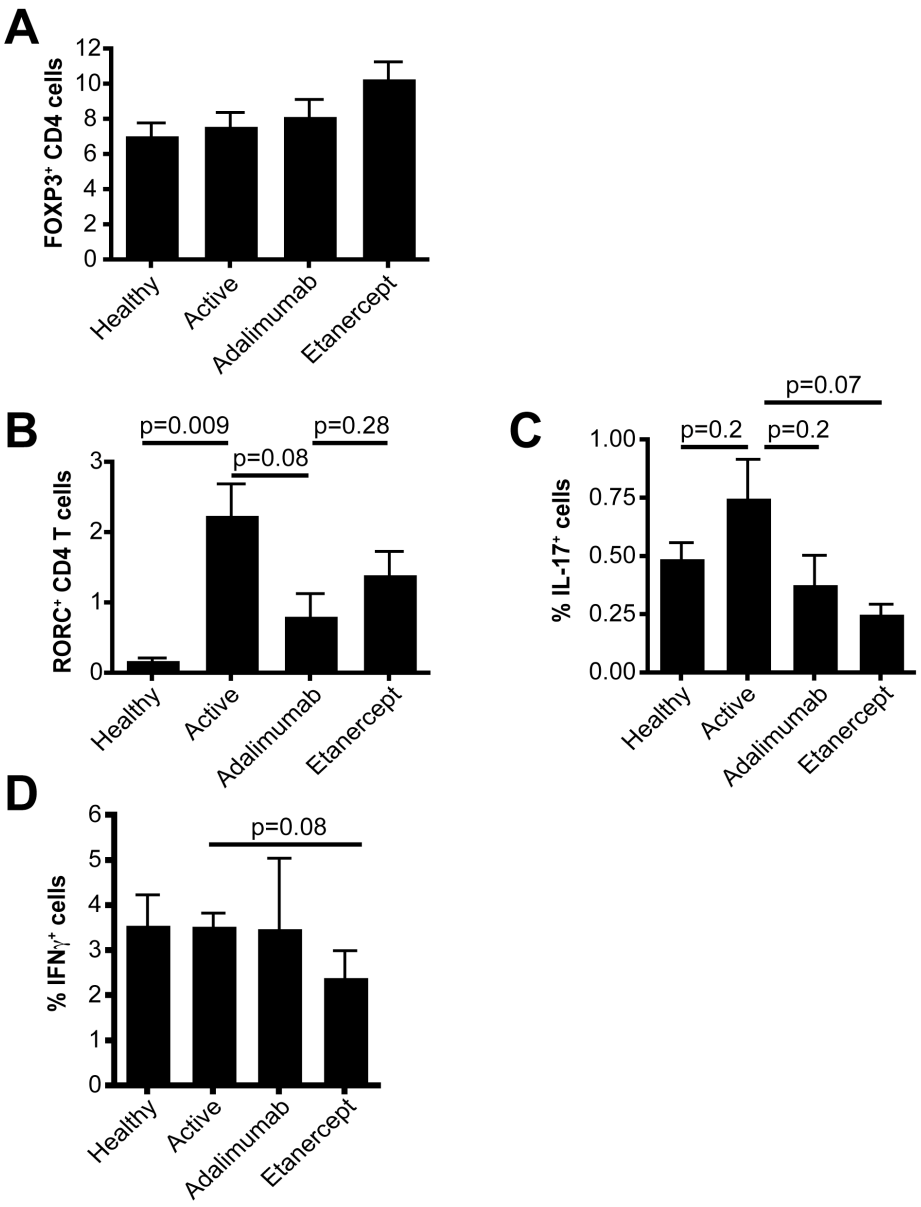


**Figure 6.1. CD4 T cells in psoriatic arthritis.**
**A.** Percentage $CD4^+FOXP3^+$ in PBMC from healthy controls, patients with active PsA and PsA patients responding to adalimumab or etanercept (*n=8*). Bars represent the mean±SE. **B.** PBMC from healthy controls (*n=9*), patients with active PsA (*n=12*) and PsA patients responding to adalimumab or etanercept (*n=5*) were stained *ex vivo* for CD4 and RORC. Bars indicate mean percentage $CD4^+RORC^+$ cells ±SE. **C.** *Ex vivo* PBMC from healthy controls (*n=6*), patients with active PsA (*n=12*) and patients treated with adalimumab or etanercept (*n=5*) were stimulated for 4 hours with PMA before staining with IL-17. Graphs show percentage $IL\text{-}17^+$ lymphocytes. Bars represent mean ±SE **D.** *Ex vivo* PBMC were stimulated as in **'C'** and stained for IFNγ. Graphs show percentage $IFN\gamma^+$ lymphocytes. Healthy (*n=6)*, active (*n=12)*, adalimumab and etanercept (*n=3)*. Bars represent mean IFNγ production ±SE.

## 6.3 Proliferation and cell death in patients with PsA

We next examined proliferation and cell death in patients with PsA. There were no differences in proliferation between healthy controls, patients with active PsA and PsA patients treated with adalimumab or etanercept in either total $CD4^+$ T cells (Figure 6.2 A) or $FOXP3^+$ T cells (Figure 6.2 B). When cell death was determined there was no significant difference between healthy controls and patients with active PsA however, there was a trend for etanercept treated PsA patients to have increased levels of total cell death after therapy (Figure 6.2 C). Moreover, similar to their RA counterparts, patients with active PsA have a significant reduction in monocyte cell death compared to healthy controls ($p=0.02$)(Figure 6.2 D).

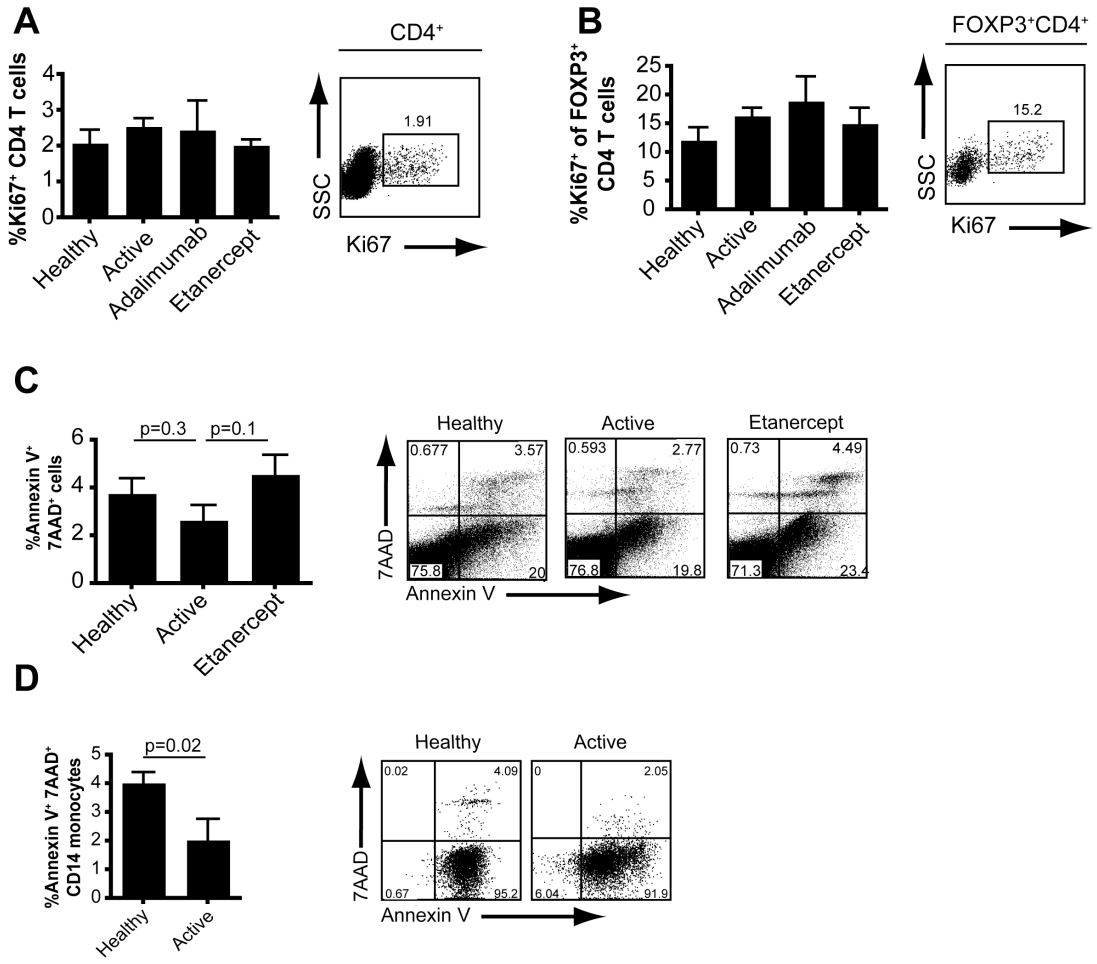


**Figure 6.2. Proliferation and cell death in psoriatic arthritis**

**(A-B).** *Ex vivo* PBMC from healthy controls (*n=7*), patients with active PsA (*n=12*) and PsA patients responding to adalimumab (*n=3*) or etanercept (*n=4*) were stained with CD4, FOXP3 and Ki67. **A.** Percentage Ki67$^+$ CD4 cells. Bars represent the mean±SE. An example FACS plot is shown. **B.** Percentage Ki67$^+$ cells in the CD4$^+$FOXP3$^+$ population. Bars represent the mean±SE. An example FACS plot is shown. **C.** *Ex vivo* PBMC (healthy *n=10*, active *n=5*, etanercept *n= 5*) were stained with Annexin V and 7AAD. A 'live' gate was not used for analysis. Graph shows percentage Annexin V$^+$7AAD$^+$ cells. Bars represent the mean±SE. Representative FACS plots are shown. **D.** Percentage Annexin$^+$7AAD$^+$ cells in the CD14$^+$ population (healthy n=9, active n=5). A 'live' gate was not used for analysis. Bars represent the mean±SE. Representative FACS plots are shown.

## 6.4 Treg from PsA patients treated with anti-TNF are unable to suppress IL-17 production from PBMC

Little is known about the functional characteristics of Treg in PsA, although regulatory T cells from patients with psoriasis are known to be defective [444]. It is known that IL-17 and IL-22 are heavily involved in the aetiology of psoriasis [445] and that 70% of patients with PsA develop arthritis after the onset of skin disease [446]. Therefore, utilising the depletion approach described in section 4.2, it was investigated if Treg from PsA patients responding to anti-TNF therapy were capable of suppressing IL-17. By ELISA there was an overall increase in IL-17 production in patients with psoriatic arthritis compared to healthy controls, or anti-TNF treated PsA patients, matching the Th17 data presented in figures 6.1 B and C (Figure 6.3. B). However, both flow cytometric and ELISA data showed that, like healthy controls, patients with active PsA and PsA patients treated with both anti-TNF therapies showed no increase in IL-17 upon the depletion of Treg, suggesting an inability to suppress this cytokine (Figure 6.3 A-B).

## 6.5 Suppression of IL-22 by Treg from patients with PsA

In order to determine the ability of Treg from patients with PsA to suppress IL-22, Treg were depleted from whole PBMC as previously described. Cells from healthy controls showed an increase in IL-22 production upon the depletion of Treg, suggesting that healthy Treg can suppress IL-22. Surprisingly, in patients with active PsA the production of IL-22 increased substantially following the depletion of Treg. This suggests that PBMC from patients with active PsA have the potential to produce large quantities of IL-22 but that this is readily suppressed by Treg from these

patients. In PsA patients treated with adalimumab the data matched that of healthy controls. In etanercept treated patients, there was no evidence of an increase in IL-22 following the depletion of Treg, suggesting that Treg from etanercept treated patients are unable to suppress IL-22 (Figure 6.3 C).

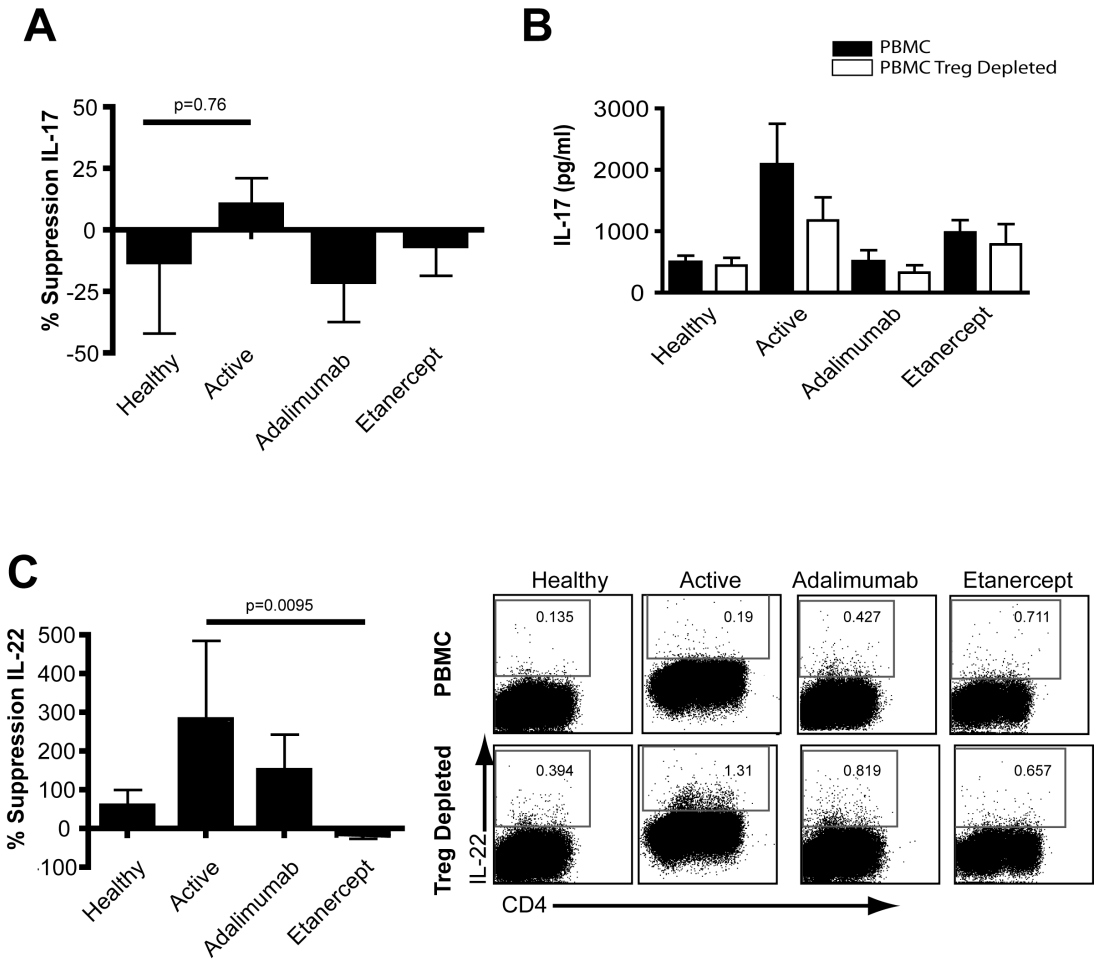


**Figure 6.3. Modulation of IL-17 and IL-22 production by Treg in PsA patients before and after anti-TNF therapy.**

PBMC were separated into 2 fractions, one with whole PBMC and the other depleted of $CD4^{+}CD25^{+}CD127^{-}$ Treg. Cells were cultured for 3 days with soluble anti-CD3 and anti-CD28. After 3 days cells were stimulated with PMA, Ionomycin and Golgi Stop. **A.** Cells from healthy controls (*n=4*); patients with active PsA (*n=3*) and patients responding to adalimumab (*n=3*) or etanercept (*n=3*) were stained with flow cytometry antibodies for IL-17. Bars represent the mean percentage change in IL-17 production between PBMC and PBMC depleted of Treg (%IL-17 suppression). **B.** Supernatants were assayed for IL-17 using ELISA. Bars represent the mean±SE. 'n' numbers as for part A **C.** Cells were stained with flow cytometry antibodies for IL-22. Healthy *n=2*, PsA *n=6*, adalimumab *n=4*, etanercept *n=4*. Bars represent the mean of percentage difference in IL-22 production between PBMC and PBMC depleted of Treg. Representative FACS plots are shown.

Summary:

Compared to healthy controls, there were no differences in the percentage of circulating Treg in patients with active PsA or PsA patients responding to adalimumab or etanercept. In contrast, there was a sharp increase in Th17 cells in patients with active PsA compared to healthy controls and this was reduced in PsA patients responding to both adalimumab and etanercept therapy. There were no differences in *ex vivo* IFNγ production between healthy controls, patients with active PsA and PsA patients responding to anti-TNF. There were also no differences in the proliferation of CD4 T cells or Treg. Like RA patients, cell death was lower in patients with active PsA and this reached significance when monocyte cell death was determined.

Neither patients with active PsA nor patients responding to adalimumab or etanercept demonstrated an ability to suppress IL-17 production from whole PBMC. In contrast, Treg from patients with active PsA showed a clear capacity to restrain IL-22 production. This suppression of IL-22 was not altered by therapy with adalimumab but did appear to be impaired by therapy with etanercept.

# Chapter 7

# Discussion

## 7.1 The distinctive properties of regulatory T cells from rheumatoid arthritis patients treated with adalimumab

### 7.1.1 The suppression of Th17 responses

The major finding from this work is that Th17 cell responses can be suppressed by Treg from RA patients responding to adalimumab (AdTreg) via the control of monocyte-derived IL-6 production (Chapter 4). Suppression of IL-17 was not apparent when Treg from healthy individuals were analysed (Figure 4.4) suggesting, as previously published, that this cytokine can be resistant to the effects of Treg [37]. The potential of anti-TNF monoclonal antibody (mAb) therapy to regulate this highly inflammatory pathway is reflected in the shift away from Th17 cells *ex vivo* in RA patients treated with adalimumab (Figure 3.12). However, responder T cells from adalimumab treated RA patients still maintain the capacity to produce high levels of IL-17. In the absence of Treg, cultures of responder T cells from adalimumab treated patients produce much more IL-17 than healthy controls or patients treated with etanercept. This is dramatically reduced by the addition of AdTreg (Figure 4.4), highlighting the potent modulation of Th17 responses by these cells.

Additionally, the data presented here show that in RA patients, the suppression of the Th17 associated cytokine IL-22 [195, 232, 447] mirrors the suppression of IFNγ rather than IL-17 (Figure 4.2). Indeed, the depletion of Treg from whole PBMC of healthy individuals resulted in an increase in IL-22, suggesting that healthy Treg can

effectively suppress this cytokine. However, in healthy controls levels of IL-22 were very low (Figure 4.2). In patients with active RA, the removal of Treg resulted in levels of IL-22 that were similar or reduced, suggesting that Treg from these patients are not capable of suppressing IL-22. Like the capacity to suppress IFNγ, suppression of IL-22 was restored in RA patients treated with adalimumab, but not patients treated with etanercept. The differential regulation of IL-17 and IL-22 is surprising considering that these cytokines are both known to be produced by Th17 cells. However, a distinct population of Th22 cells, producing IL-22 but not IL-17 has recently been described [448]. The production of these cytokines by different cell subsets may explain how healthy Treg can suppress IL-22 but not IL-17 production.

### 7.1.2 An intrinsic suppression of IL-17 production

When Treg were added to cultures of responder T cells and monocytes from patients with active RA and RA patients treated with etanercept the production of IL-17 increased (Figures 4.2 and 4.4), suggesting that Treg from these patients make IL-17. Treg production of IL-17 has been well documented [217-219], as has the propensity of Treg to convert to Th17 cells [219, 449, 450]. Indeed, work in this laboratory has shown that under inflammatory conditions Treg display increased production of IL-17. However, AdTreg made less IL-17 after 5 days than patients with active disease (RATreg) or those treated with etanercept (EtTreg) [451]. Thus, Treg from adalimumab treated RA patients are distinctive from Treg in healthy controls and other RA patients in that they show both an intrinsic and an extrinsic suppression of IL-17, which results in a clear reduction in overall IL-17 levels.

### 7.1.3 Regulatory T cells from rheumatoid arthritis patients treated with adalimumab share phenotypic similarities with induced regulatory T cells

Previous data from this group showed that the chimeric anti-TNF mAb, infliximab, induced a population of functional CD62L negative Treg in patients with RA [86]. In this study it has been shown that adalimumab treated RA patients also have an increase in CD62L negative Treg. Furthermore, these Treg express lower levels of Helios, the marker of thymic Treg, compared to Treg from patients with active RA (RATreg) (Figure 3.3). Thus, AdTreg bear distinctive hallmarks of induced Treg (iTreg). It is interesting that the CD62L negative AdTreg and the Helios negative AdTreg are not entirely overlapping populations, perhaps suggesting that double negativity for these markers offers the best method for the *ex vivo* identification of Treg induced by adalimumab therapy.

There is controversy however, surrounding Helios as a marker of thymic Treg (discussed in the introduction section 1.1.3.3). Furthermore, a loss of CD62L expression is not unique to iTreg but is associated with a memory T cell phenotype [452]. So these markers do not prove that Treg have been induced by therapy. However, the increase in Treg in RA patients treated with adalimumab is not explained by proliferation of existing Treg, as levels of Ki67 are similar before and after therapy (Figure 3.4). Moreover, these cells suppress IL-17 in contrast to functional Treg from healthy controls (HTreg) and Treg from patients with active RA (RATreg) (Figure 4.4). Thus, a population of Treg is present in RA patients treated with adalimumab that is not present in any other patient group. This supports previously published data demonstrating that anti-TNF mAb therapy in RA induces a population of functionally and phenotypically distinct Treg [86]. It is tempting to

speculate that the disease exacerbation that eventually occurs following cessation of anti-TNF therapy may be delayed due to the presence of AdTreg. Indeed, one of the first studies to examine recurrence of disease activity once anti-TNF therapy was stopped, found that those patients treated with infliximab, did not flare as rapidly as those that received only methotrexate [453]. Thus, it would be interesting to discover if patients treated with adalimumab and etanercept flare equally after cessation of anti-TNF therapy.

## 7.2 How do regulatory T cells from adalimumab treated rheumatoid arthritis patients suppress IL-17?

### 7.2.1 Monocyte suppression

It is interesting that the Treg induced by adalimumab therapy in RA control Th1 and Th17 pathways through separate mechanisms. Treg are thought to use multiple pathways to control target cells, but these data demonstrates for the first time that independent modalities of suppression can be employed to regulate different human effector T cell lineages. IL-10 and TGFβ were required for the suppression of Th1 responses (Figures 4.1 and 4.3). The requirement for IL-10 was highlighted by the impairment of IFNγ suppression upon the blockade of IL-10 (Figure 4.5) and the increased IL-10 production upon the addition of AdTreg to autologous responder T cells (Figure 4.1). However, by flow cytometry, IL-10 production could not be detected from Treg (Figure 3.8). Thus, these data do not exclude the possibility that the IL-10 required for the suppression of IFNγ is made by responder T cells in response to a signal from AdTreg. In contrast, the neutralisation of IL-10 and TGF-β had no effect on the ability of AdTreg to suppress IL-17 (Figure 4.5). Rather, an IL-

10 and TGF-β independent modulation of IL-6 appeared to be pivotal. The pattern of IL-6 suppression by AdTreg exactly matched that of IL-17 (Figure 4.12) and the neutralisation of IL-6 in cultures of responder T cells and monocytes from adalimumab treated RA patients imitated the IL-17 suppressing abilities of AdTreg. Moreover, addition of IL-6 reversed the ability of AdTreg to suppress Th17 cells (Figure 4.13). The capacity of AdTreg to directly suppress monocyte IL-6 production was confirmed by showing that monocytes from patients with active RA made less IL-6 when cultured with AdTreg than with autologous RATreg or Treg from RA patients treated with etanercept (EtTreg) .

Previous publications have shown that Treg suppression of monocytes is partly contact dependent [441]. However, inhibition of IL-6 and IL-17 was not impaired when Treg contact was prevented in a transwell system (Figure 4.14). Thus, suppression of these responses must be mediated by a soluble factor secreted by AdTreg. It is still unclear what this soluble factor might be; IL-35 production by Treg (Figure 4.11) may offer an explanation, though more experiments are needed to confirm a role. The blockade of IL-10 was shown to be unimportant for the suppression of Th17 cells (Figure 4.5), however other Th2 cytokines such as IL-4 or IL-13 could suppress the production of IL-17 by altering the balance of cytokines *in vivo*. Indeed IL-4 can suppress disease in mice with collagen-induced arthritis (CIA) [454]. Moreover, IL-4 and IL-13 have been detected in human Treg [441, 455, 456]. However, Sarkar et al. show that IL-4 does not directly modulate Th17 cells in CIA and is more important for the suppression of Th1 cells. Thus perhaps in humans too, Th2 cytokines predominantly modulate Th1 responses, explaining the inability of IL-10 to suppress Th17 responses from RA patients treated with adalimumab.

Recently it has been shown that during septic shock, human Treg can inhibit monocyte survival through the production of a soluble factor that activates the Fas/FasL pathway [457]. Not only does this offer an explanation for the AdTreg capacity to suppress monocyte-derived IL-6 (Figure 4.12), but the ability of AdTreg to trigger the apoptosis of monocytes offers an explanation as to why adalimumab but not etanercept treated RA patients show an increase in monocyte cell death (Figure 3.6). It has recently been published that in healthy individuals, responder T cells and not Treg are responsible for Fas/FasL driven monocyte apoptosis. The authors show that this mechanism of monocyte apoptosis is defective in patients with RA [458]. AdTreg have been shown to be functionally distinct from HTreg, so it would be interesting to investigate if the capacity of AdTreg to suppress IL-6 and IL-17 is linked to the killing of monocytes in culture.

### 7.2.2 Activation of the phospho-STAT3 pathway

It is intriguing that suppression of IL-17 in RA patients treated with adalimumab is mediated via the suppression of IL-6, more so when AdTreg show increased activation of STAT3, a component the IL-6 signalling cascade, upon stimulation compared to their counterparts that do not suppress IL-17 (Figures 4.8 and 4.9). STAT3 is considered to be a marker of Th17 cell lineage but, in a decisive shift in the accepted paradigm, STAT3 expression in murine Treg was shown to be essential for the capacity of these cells to suppress IL-17 [436]. Moreover, it has been shown that the ablation of STAT3 during the induction of Treg from murine responder T cells prevented the acquisition of suppressive function [459]. This implicates

STAT3, a signalling molecule known to drive Th17 cell differentiation, in the suppression of IL-17 and the function of iTreg.

Interestingly, recent evidence suggests that Treg are generated and function in a manner defined by their microenvironment. For example, IRF4, a Th2 associated transcription factor can form complexes with FOXP3 in Treg to mediate suppression of Th2 driven inflammation. In the absence IRF4 expression in Treg, there is an unprovoked Th2 driven pathology [460]. Similarly, expression of T-bet in Treg allows them to express CXCR3 to trigger accumulation at the site of an on-going Th1 response [461]. Thus, it is tempting to speculate that the presence of large quantities of IL-6 and IL-17 in RA patients during the adalimumab-driven induction of Treg defines the phenotypic and functional properties of the cell that is induced. That is a Treg that suppresses IL-6 and IL-17 responses in a STAT3-dependent manner.

A recent study by Chaudhry et al. suggests that it is not IL-6 that activates STAT3 in Treg, but rather, IL-10 via the high expression of IL-10R on Treg [462]. This IL-10-dependent model of STAT3 mediated suppression is supported by some of the data presented in this study. For example, Treg from adalimumab treated patients stimulated with IL-6 do not show the same pattern of pSTAT3 induction as those stimulated via the TCR (Figure 4.9). Furthermore, AdTreg may make IL-10, which could act in an autocrine manner upon the cell to activate STAT3. However, in contrast to the data presented by Chaudhry et al., IL-10 is not required for the suppression of IL-17 by AdTreg (Figure 4.5). It is possible that the mechanism of STAT3 activation differs in human and murine Treg. Indeed, a number of factors

activate the STAT3 pathway and could be important for the capacity of AdTreg to suppress Th17 responses. One such factor may be IL-35 which, like IL-6, signals to target cells using the receptor molecule gp130. Although a recent study in mice has shown that IL-35 activates STAT1 and STAT4 but not STAT3 [463].

The ideal experiment to determine a role for pSTAT3 in the function of AdTreg would be to use a STAT3 inhibitor to effectively impair suppressive function and then replicate this data with siRNA knockdown of STAT3 in AdTreg. In the absence of this functional readout, the initial experiments examining the pSTAT3 expression in TCR stimulated Treg (Figures 4.8 and 4.9) were designed simply to determine if there were differences in pSTAT3 expression upon activation of Treg. In these experiments IL-6 was routinely used as a positive control to drive the phosphorylation of STAT3. Regardless of whether pSer727 or pTyr705 was used as a marker of STAT3 phosphorylation, the MFI of IL-6 stimulated cells was higher than unstimulated cells (Figure 4.9 A) or cells stimulated with anti-CD3 and anti-CD28 (Figure 4.8 B and Figure 4.9B). Moreover, the shift in MFI seen upon stimulation with IL-6 was reversed in the presence of a STAT3 inhibitor (Figure 4.10 A) and the fold induction of pSTAT3 was shown to be reduced in a concentration dependent manner upon treatment with inhibitor (Figure 4.10 D). Whilst these changes seem consistent, the MFI of both pTyr705 and pSer727 were low and the fold change in pSTAT3 was small. In order to make this a more robust positive control the assays could be supplemented with soluble IL-6 receptor in order to amplify the signal via gp130.

## 7.3 The role of interleukin-6 in Th17 cell differentiation: Insight from tocilizumab treated rheumatoid arthritis patients

The importance of IL-6, but not IL-1, for the modulation of Th17 cells in patients with RA was unexpected (Figure 4.12). Both cytokines have been shown to be important for human Th17 cell differentiation [190, 195, 439, 464]. However, the data in adalimumab treated RA patients shows that even in the presence of IL-1β, the neutralisation of IL-6 reduces IL-17 production (Figure 4.13 B).

Thus it was predicted that RA patients treated with tocilizumab, the anti-IL-6 receptor antibody, would have a reduction in Th17 cells. Furthermore, due to the reciprocal relationship between Treg and Th17 cells, it was hypothesised that these patients would demonstrate an increase in Treg. Surprisingly, whilst the levels of Treg were similar in healthy controls and RA patients examined before anti-TNF therapy (Figure 3.1), the percentage of peripheral Treg was much higher in RA patients before tocilizumab therapy than in healthy controls (Figure 5.1 A). This is likely to be explained by the fact that patients commencing tocilizumab have been treated with multiple DMARDS and biologic agents, including up to 3 different anti-TNF therapies and perhaps also rituximab. Six weeks after the first infusion with tocilizumab there were few differences in peripheral Treg and Th17 cells (Figure 5.1). However, there was a reduction in the production of the Th17 cell cytokine, IL-21, by CD4 T cells of patients treated with tocilizumab (Figure 5.5 B). This may suggest that there is some inhibition of the Th17 cell profile in patients treated with tocilizumab. Indeed, differences in the balance of Treg and Th17 cells have recently been observed 12 weeks after therapy [465], which suggests that there may be more notable changes in these populations at a later time point.

## 7.4 Tocilizumab therapy: Targeting B cells?

In contrast to the T cell data, the neutralisation of IL-6 signalling altered the B cell populations in 3 patients after 6 weeks of therapy. The data presented here show a reduction in the number of $CD27^{+}$ $IgD^{-}$ memory B cells (Figure 5.5A), mirroring data which has previously been published [384]. Furthermore, these patients also demonstrated a reduction in the percentage of plasma cells (Figure 5.5A). Interestingly, IL-21 is thought to be important for plasma cell differentiation [466]. Thus, the reduction in IL-21 production from CD4 T cells after tocilizumab therapy (Figure 5.5 B) could offer an explanation for the reduction in this B cell subset. This may suggest that tocilizumab therapy could be appropriate for patients with B cell driven pathology and offers a benefit over rituximab in that it may target the primary producers of pathogenic autoantibody.

## 7.5 Immunopathogenic features of psoriatic arthritis

There have been few studies investigating the immunopathogenesis of psoriatic arthritis (PsA). Here it has been shown for the first time that compared to healthy controls there is no alteration in peripheral Treg levels in patients with active PsA (Figure 6.1 A). Moreover, the ability of Treg from patients with active PsA to suppress IL-22 and IL-17 is similar to Treg from healthy controls (Figure 6.3), suggesting that there may be no dysfunction in Treg from patients with PsA. This may explain why the induction of Treg in PsA patients treated with anti-TNF is not a feature of therapeutic response (Figure 6.1 A).

Unlike RA, the T cell repertoire in the synovium of PsA patients is skewed towards CD8 rather than CD4 T cells [416]. Indeed, there are a number of features of PsA

that distinguish disease from active RA and suggest a greater similarity to spondyloarthropathies such as ankylosing spondylitis (AS). In particular the IL-23 pathway, perhaps through the control of Th17 cells, has been implicated in the pathogenesis of AS [467] and also psoriasis [400]. The data presented here shows that IL-17 production from whole PBMC (Figure 6.3 B), levels of $RORC^+$ Th17 cells (Figure 6.1 B) and levels of $IL\text{-}17^+$ cells *ex vivo* (Figure 6.1 C) were much higher in patients with active PsA than patients with active RA, thus suggesting a key role for IL-17 as pro-inflammatory mediator in PsA. Interestingly, a drop in the levels of Th17 cells was associated with response to both adalimumab and etanercept therapy (Figure 6.1 C). Elucidating how etanercept can reduce Th17 levels in PsA but not RA might provide further insight into the mechanism of action of etanercept therapy. Due to the differences between PsA and RA, in addition to investigating CD4 T cells in the pathogenesis of PsA, future work should examine $CD8^+$ T cell cytokine production or cytotoxic activity and whether these responses are adequately controlled by Treg from patients with active PsA.

### 7.5.1 The role of IL-22 in the pathogenesis of psoriatic arthritis

In recent years, lymphocyte-derived IL-22 has been shown to be heavily involved in inflammatory skin disorders including psoriasis [468-470] and PsA [410]. So it was hypothesised that the amelioration of disease seen in psoriatic arthritis patients treated with anti-TNF may be due to the altered capacity of Treg from these patients to suppress of IL-22. In contrast to patients with RA (Figure 4.2), Treg from active PsA patients were shown to be potent suppressors of IL-22 as levels of IL-22 were dramatically increased upon the depletion of Treg from cultures of whole PBMC.

Similarly, Treg from healthy controls and PsA patients responding to adalimumab could suppress IL-22. Thus, it was surprising that the depletion of Treg from the PBMC of etanercept treated PsA patients did not affect the production of IL-22 (Figure 6.3 C). This suggests that Treg from PsA patients responding to etanercept have 'lost' the capacity to suppress IL-22 and perhaps indicates that IL-22 is not pro-inflammatory in these patients.

Under some conditions IL-22 has been shown to have anti-inflammatory and tissue reparative properties [226, 291, 293, 294, 307-310]. However, IL-22 has been shown to be pro-inflammatory in skin conditions [290, 296, 300, 471], including PsA [410] and all evidence suggests that IL-22 is pro-inflammatory in RA [208, 302-304]. Although, it has been suggested that the presence of IL-17 defines the pro- versus anti-inflammatory effects of IL-22 [316]. Thus, perhaps the reduction in Th17 cells in PsA patients treated with etanercept favours the anti-inflammatory properties of IL-22 and promotes the repair of tissue damaged by inflammation. How, or why, anti-TNF therapy would impair Treg suppression is unclear and why this is seen in etanercept treated PsA patients but not adalimumab treated PsA patients remains to be elucidated. It may be that Treg from etanercept treated patients continue to suppress CD4 T cell production of IL-22 but therapy triggers the production of IL-22 by another cell subset that is not so readily suppressed by Treg. Co-culture experiments with different cell subsets would offer some clarification of the role of IL-22 in PsA and the capacity of Treg to suppress its production.

## 7.6 Anti-TNF therapy induces a different response in rheumatoid arthritis and psoriatic arthritis

If adalimumab treatment ameliorates disease in RA, perhaps in part through the induction of Treg, then one would predict that this anti-TNF agent would work in another disease setting via the same mechanism. However, the data presented in this study reveals that after treatment with anti-TNF, patients with PsA do not have an increase in peripheral Treg (Figure 6.1 A) and Treg from PsA patients do not acquire the ability to suppress IL-17 (Figure 6.3).

## 7.7 Potential models for the induction of regulatory T cells by anti-TNF monoclonal antibody therapy in rheumatoid arthritis.

It is unclear why adalimumab did not induce Treg in patients with PsA when it has been shown to do so in other disease settings. Likewise there is no clear explanation as to why adalimumab induces Treg in patients with RA whilst etanercept treated RA patients show no increase in Treg (Figure 3.1) and a continued Treg defect despite responding to therapy (Figures 4.1, 4.3 and 4.4). In the following sections a number of models for the adalimumab driven induction of Treg in patients with RA are discussed and their validity is assessed based on the data presented in this study.

### 7.7.1 The neutralisation of TNF

Studies examining the effect of TNF on Treg have generally proposed a model whereby TNF can signal to, and block, the suppressive capacity of Treg. Thus, upon the neutralisation of TNF and in the absence of this signal, Treg function is restored [130, 472]. Indeed, this effect of TNF on Treg was shown to be mediated via

recruitment of PKC-θ to the immune synapse. In Treg treated with TNF, a reduction in suppressive function was associated with recruitment of PKC-θ to the immune synapse and in patients with active RA, treatment of Treg with a PKC-θ inhibitor restored suppressive function [129, 130]. However, if TNF was sufficient to impair the activity of Treg and its blockade reversed this effect, then both anti-TNF therapies would be equally effective in restoring Treg function. Rather, the data presented here suggests that the Treg defect in patients with active RA is not simply a direct result of the presence of inflammation because, despite an equivalent clinical response, EtTreg remain defective (Figures 4.1, 4.3 and 4.4). Moreover, the persisting defect in EtTreg suggests that the defective suppression by RATreg is not simply explained by the inflammation associated with RA making responder T cells less susceptible to suppression. This supports previous findings by this group [124] and others [129, 130] in which cross-over experiments showed that responder T cells from patients with active RA are not resistant to Treg suppression. Therefore, the inability of Treg to control inflammation in patients with active RA is due to a defect in Treg rather than unresponsiveness of responder T cells.

In a recent report, anti-TNF therapy was shown to down-regulate genes associated with TNF pathways regardless of response to therapy. This led the authors to propose a model whereby clinical response to therapy is associated with TNF-independent pathways and that these pathways might be specific to the individual [473]. Indeed, the observations in this study suggest that anti-TNF monoclonal antibodies and etanercept exert their therapeutic effects in RA through distinct mechanisms which are seemingly independent of the blockade of TNF (Figure 3.1) and may underlie the

fact that patients with RA who fail one form of anti-TNF can respond to another [474, 475]

### 7.7.2 Differential binding of adalimumab and etanercept to membrane bound TNF

Whilst the differences in Treg induction between therapies and disease do not define a common role for TNF in Treg biology, establishing that the induction of Treg is specific to RA patients treated with adalimumab might provide some insight into the requirements for the peripheral generation of Treg. One of the explanations that may account for the difference in Treg induction between the anti-TNF antibodies and etanercept in RA is their respective binding efficacies for soluble and transmembrane TNF (mTNF). Both infliximab and etanercept were shown to bind to mTNF and were both able to prevent mTNF-mediated activation of human endothelial cells. However, infliximab binding to mTNF was more effective in blocking the activation of endothelial cells than etanercept, and infliximab association with mTNF had a higher avidity and was more stable than etanercept. This led the authors to conclude that infliximab mediates a more complete and sustained neutralisation of TNF than etanercept [366].

Thus, perhaps the induction of Treg in RA patients treated with the anti-TNF mAb (Figure 3.1) is mediated via a sustained neutralisation of TNF, as opposed to a substantial yet incomplete neutralisation of TNF in RA patients treated with etanercept. However, in PsA patients these differences in the ability of anti-TNF therapies to bind and neutralise TNF would be the same and yet there is no induction of Treg in adalimumab treated PsA patients (Figure 6.1 A). Recently, a number of

studies have suggested that TNF and signalling through TNFR2, is fundamentally required for the induction of Treg. Moreover, it has been suggested that anti-TNF therapies impair this induction [476]. In this TNF-driven model of Treg induction, the more transient neutralisation of TNF in etanercept treated patients would be more likely to result in an induction of Treg. Thus, a disparity in the neutralisation of TNF may not be sufficient to explain the induction of Treg in RA patients treated with adalimumab.

#### *7.7.2.1 Alterations in apoptosis induce an immunoregulatory phenotype.*

Mitoma et al. showed that whilst infliximab and etanercept could bind to mTNF and drive the up-regulation of E-selectin in a human Jurkat T cell line transfected with mTNF, only infliximab could induce apoptosis. Infliximab-mediated apoptosis was reversed by a mutation of mTNF that prevented signal transduction [477]. These data suggests that anti-TNF antibodies preferentially signal to cells that express mTNF. This could have a number of implications. Firstly it offers an explanation as to why monocytes, the primary population of cells expressing mTNF [478], from RA patients treated with adalimumab have an increase in monocyte cell death but etanercept treated patients do not (Figure 3.6). Secondly, it is known that an abundance of apoptotic cells promotes tolerance and can contribute to the induction of Treg. Indeed it has been shown that the uptake of apoptotic DCs by live immature DCs prevents maturation and the production of inflammatory cytokines whilst stimulating the production of TGF-β and the induction of $FOXP3^+$ Treg [479]. Moreover, murine anti-CD3 therapy, a T cell depleting biologic agent, was shown to mediate the induction of Treg via phagocyte production of TGF-β upon the uptake of

apoptotic T cells [480]. Thus, perhaps in RA patients treated with adalimumab there is an increase in apoptotic monocytes that are taken up by phagocytes and in turn produce anti-inflammatory cytokines that can drive the induction of Treg.

#### *7.7.2.2 The activation of p38 downstream of membrane-bound TNF*

In addition to driving apoptosis, reverse signalling through mTNF has been shown to activate the MAP kinase pathway [481]. The p38 MAP kinases regulate cytokine production in response to cytokine stimulation. *In vitro* treatment of the mucosa of Crohn's disease patients with infliximab was shown to transiently induce p38 expression [482]. Moreover, it was shown that etanercept therapy failed to activate this pathway [483]. Thus differential activation of the p38 pathway could provide an explanation for the differences observed in RA patients treated with adalimumab. This may also provide a link between the purported requirement of TNF for the induction of Treg [476] and the induction of Treg by antibodies which neutralise TNF. P38 can drive the production of TNF [484]. Perhaps anti-TNF antibodies bind to mTNF on the surface of monocytes and via p38 initiate a transient burst of TNF production. This may act locally upon responder T cells to induce a population of Treg. However, this theory does not explain the capacity of infliximab to induce Treg from isolated responder T cells *in vitro* [86]. Interestingly, activated T cells have been shown to express mTNF [485]. Thus, reverse signalling through mTNF on activated responder T cells might initiate a burst of TNF production from the T cell that is used in an autocrine manner to drive the induction of Treg. A lower expression of mTNF on the monocytes or T cells of patients with PsA may explain the inability of adalimumab therapy to induce Treg in these patients.

## 7.8 What is the mechanism of action of etanercept?

The lack of functional Treg in RA patients treated with etanercept is discordant with the high levels of TGF-β expressed on both $FOXP3^+$ and $FOXP3^-$ CD4 T cells in some patients (Figure 3.7). Furthermore, the expression of TGF-β and the production of IL-10 by monocytes (Figure 3.10) and B cells (Figure 3.9) from etanercept treated RA patients should create ideal conditions for the induction of Treg and yet there is no evidence of such an induction (Figure 3.1). Thus, perhaps the IL-10 and TGF-β produced by monocytes and T cells from RA patients treated with etanercept support the restoration of tolerance in these patients without the induction of Treg. Indeed, both IL-10 and TGF-β have been shown to suppress inflammation[486]. This theory is supported by ELISA data in which the production of IL-17 and IL-6 from cultures of responder T cells and monocytes from etanercept treated RA patients remains very low after 5 days (Figures 4.4 B and 4.12 B). It is not clear how etanercept might mediate this induction of tolerance and why it would not be imitated by anti-TNF antibodies. Thus, in RA, etanercept as well as adalimumab has distinct effects on immune regulation and the challenge will be to discover how etanercept mediates these effects.

## 7.9 Clinical implications of the differences in anti-TNF therapy

Ostensibly, blockade of both TNF and IL-17 pathways in patients with RA could confer a therapeutic advantage upon adalimumab therapy, but could also render the patient more prone to infection given the importance of these cytokines for host defence. RA patients treated with either adalimumab or infliximab have a 7-17 fold higher risk of developing a *Mycobacterium tuberculosis* (TB) infection than RA patients treated with etanercept, most likely due to re-activation of the latent

bacterium [365]. TB is a granulomatous infection where macrophages and inflammatory cells are recruited to the site of infection to form a barrier around the mycobacterium. It is known that IFNγ production is particularly implicated in immunity against TB, as animals which lack the ability to make this cytokine are more susceptible to infection [487, 488]. Although IL-17 may have a limited role in primary infection, it appears to control granuloma formation and bacterial burden, both of which would be critical to TB reactivation [489, 490]. Healthy Treg can prevent an effective immune response to TB [491-493], and therefore it is possible that AdTreg, through inhibition of Th1 and Th17 responses, would have a profound effect on TB immunity. In contrast, regulatory T cells from RA patients treated with etanercept are unable to modulate either Th1 or Th17 cells (Figures 4.1-4.4), consistent with the finding that this agent has little impact on the incidence of TB [365]. The challenge will be to refine anti-TNF antibody therapy such that these induced Treg can permit cessation of treatment for these inflammatory diseases, thereby inducing tolerance, but also protect patients from developing TB.

Similarly, it is known that anti-TNF mAb therapy, but not etanercept, is effective in granulomatous diseases such as Crohn's disease [361, 362] and sarcoidosis [363, 364]. The key difference between adalimumab and etanercept therapy in RA is the capacity of adalimumab therapy to drive the induction of Treg. Like patients with RA, it has been published that there is an increase in peripheral Treg in Crohn's disease patients treated with adalimumab [494]. Furthermore, a number of recent publications have found IL-17 secreting T cells and macrophages surround the central core of granulomas in patients with sarcoidosis [495, 496]. These cells were shown to persist in relapsed patients, but were absent in patients who recovered

[495]. This identifies Th17 cells as important in the granuloma phase of disease, as has also been published in TB [490]. Together, these reports offer a theory as to why adalimumab, but not etanercept therapy, is a suitable treatment in granulomatous diseases. Namely, that adalimumab treated patients have an induction of Treg that can suppress IL-17 producing cells surrounding the granuloma, resolving inflammation and ameliorating disease. The inability of etanercept therapy to induce IL-17 suppressing Treg, may explain the failure of this drug to treat granulomatous diseases.

## 7.10 Conclusion

In conclusion, the results presented here show that despite a similar clinical response to therapy, adalimumab, but not etanercept treated RA patients demonstrate an induction of Treg with distinct functional properties. The capacity of these Treg to suppress not only IFNγ but also potently arthritogenic Th17 cells highlights the potential of adalimumab therapy to drive a restoration of tolerance in patients with RA. In contrast to the regulation of Th1 responses, suppression of IL-17 is not dependent upon IL-10 or TGF-β production. Rather, the suppression of Th17 cells by Treg from adalimumab treated RA patients is mediated via the modulation of monocyte-derived IL-6. This capacity to control both IL-6 and IL-17 was shown to be dependent upon the Treg production of an unknown soluble factor and this thesis has identified activation of STAT3 as possible avenues for future research. Finally, it has been shown here for the first time, that patients with PsA have no numerical deficiency in Treg and that these Treg have no defect in their capacity to suppress IL-22, a cytokine known to drive inflammation in PsA. Furthermore, both adalimumab and etanercept treatment in patients with PsA can reduce circulating Th17 cell numbers which may be a contributing factor in clinical response to therapy.

This study has shown that there is substantial variation in the immunoregulatory effects of biologic therapies, even those that block the effects of the same cytokine. This variation is dependent upon the disease which is treated and the pharmacoimmunologic properties of the therapy used. The identification of these differences and the investigation into how they might contribute to therapeutic

response has formed a large part of this study. Indeed, the data presented here have provided a rationale for the therapeutic benefit of switching between different anti-TNF agents, offered an explanation as to why patients treated with anti-TNF mAb therapy have an increased risk of TB and offered insight into *in vivo* induction of Treg which could potentially be harnessed for therapy.

## 7.11 Study Limitations

The primary finding of this study was that Th17 cell responses can be controlled by AdTreg via the suppression of monocyte-derived IL-6 production. However, the data presented here shows that AdTreg can modulate IL-6 and IL-17 production and that IL-6 can regulate Th17 cells. It does not conclusively prove that AdTreg suppress Th17 cells via this mechanism and does not exclude a direct effect of AdTreg on Th17 cells. Thus, it would be interesting to establish an effective assay system without the presence of monocytes to validate these findings.

Many of the conclusions in this study are based on changes in the proportions of cells, for example the percentage of Treg (Figure 3.1) or Th17 cells (Figure 3.10). Whilst this is informative, it may not be entirely representative of the on-going changes in patients. For example, biologic therapy may drive an absolute increase in circulating lymphocytes through a redistribution of cells from the joint and thus may alter the proportions of cell subsets without changing their absolute number. This is particularly important if you see a fall in the percentage of cells in the periphery, as observed with plasma B cells after therapy with tocilizumab (Figure 5.4). This decrease could be representative of an increase in the percentage of other B cell subsets. Furthermore, if the capacity of adalimumab to drive apoptosis was targeted to cells which express mTNF (as discussed in 7.7.2.1), the Treg population may be protected whilst the absolute number of activated T cells may be reduced and this may be reflected in an increase in peripheral Treg. For this reason, examining absolute number of cells alongside the proportion of cells would provide greater insight into the immunologic changes of patients before and after immunotherapy.

Similarly, large changes in the percentage of a small subset of cells, for example IL-10 or IL-17 producers, may only be representative of a small change in absolute number of cells. Without a functional correlate it is difficult to determine what effect these small changes may have, particularly as the role of many cytokines is context dependent and thus, difficult to dissect in human subjects. When studying differences *ex vivo* in humans without context the only option is to interpret observed differences and if possible, extend this to further functional studies. As regards IL-17, the low production of this cytokine from T cells must be considered alongside the widespread expression of IL-17 rececptor [275] which would enhance its potency. Indeed, cytokine receptor expression at the site of inflammation, the capacity of cells to amplify their effects through further recruitment of cells, co-operation with other cytokines and the localisaton of cytokine producing cells will all have an effect on the potentcy of particular cytokines.

In experiments where neutralising antibodies were used, isotype controls were not applied consistently. An isotype demonstrates that any effects observed are specific to the function of the antibody and not a generic effect of adding an antibody to cells. This is particularly important in assays using monocytes because the Fc portion of antibodies can signal through Fcγ receptor on monocytes, which may skew responses. Future experiments where neutralising antibodies are used as a tool to investigate the suppressive mechanism of AdTreg should always include an isotype control condition.

In order to confirm differences between patient groups I predominantely used multiple unpaired ‘t’ tests. In all of these tests the confidence limit is 95% which

means that there is a 1 in 20 chance that there will be a false positive result (Type I error). With each additional group that is compared, the chance of a false positive result increases, for example, if 4 groups are compared there is a 4/20 chance of a Type I error. This lowers the confidence limit of the combined tests to 80%. When using multiple tests, it is possible to take into account the number of comparisons made in order to reduce the chance of a false postive outcome. One way of doing this is to apply a Bonferroni correction where the significance level is lowered relative to the number of comparisons made. So if the desired significance level of all the tests is 0.05, this number is divided by the number of groups compared. Using the previous example, this would bring the significance level to 0.0125 or 99.9% confidence that the differences observed are not as a result of a Type I error. Lowering the threshold for significance in this way does increase the chances of excluding real differences between groups (Type II error). The chances of a Type II error increase with the number of comparisons made because the significance level can become so low as to make the test irrelevant. However for a small number of multiple comparisons it provides an adequate way to reduce Type I error.

Throughout this study, patients were classified as responding if their DAS28 score fell by 1.2 and their CRP was below 5. Inflammatory markers are incorporated into the DAS score in order to give an overall impression of response. However, if a patient had a fall in DAS28 greater than 1.2 but still had an elevated CRP they were not included in the 'responder' group. These stringent classifications mean that some patients responding to therapy may have been excluded from analysis and does represent a limitation of this study. Nevertheless, these patients would not have been included in the non-responders group as 'non-responders' were classified as those

that did not have a drop of at least 1.2 in their DAS28 score. Perhaps in future studies classification criteria should be less stringent and the data presented in a manner that allows the identification of outliers and distinct groupings of patients. This may, in turn, offer greater insight into differential responses to therapy.

## 7.12 Future Work

### 7.12.1 Investigating the suppression of IL-17

In order to investigate the mechanism of Treg suppression of IL-17, I propose examining monocyte cell death in cultures of responder T cells, monocytes and Treg from RA patients treated with adalimumab. If there is an increase in monocyte cell death in these assays I would inhibit FasL on Treg or responder T cells in the presence of monocytes and determine if this alters the ability of AdTreg to suppress IL-17. Additionally, I would use a Fas agonist to activate the Fas pathway in the absence of Treg to determine if this mimicked the suppression of IL-17 by AdTreg.

I would also continue to investigate the role of IL-35 in mediating the AdTreg suppression of IL-17. If blocking IL-35 proves to impair the capacity of Treg to suppress IL-17 then it would be necessary to determine that the anti-p35 antibody was in fact blocking IL-35 and not another IL-12 family member. Conditions with and without an isotype control should prove that any observed effects of the blockade of IL-35 are independent of Fcγ receptor signalling by the neutralising antibody. Additionally, it would be interesting to elucidate the role of pSTAT3 in the function of AdTreg. Like IL-6, IL-35 uses the molecule gp130 as a receptor, thus there may be a link between the production of IL-35 and the activation of STAT3. The best approach to investigate a role for STAT3 in Treg function would be to repeat co-culture suppression assays using a STAT3 inhibitor. This should show if STAT3 is important and if it is, the regulation of this pathway could be investigated further.

### 7.12.2 How are Treg induced by anti-TNF?

First, I would examine mTNF expression levels on monocytes and responder T cells from patients with RA and PsA. Differences in expression levels of mTNF might explain differences in the action of anti-TNF mAbs in RA and PsA. Additionally, I would examine the capacity of both adalimumab and etanercept to induce apoptosis *in vitro*. Depending on the results, it would be interesting to see if apoptotic monocytes from these assays have the capacity to differentially induce Treg when they are taken up by phagocytes.

In order to determine whether the anti-TNF molecules differentially bind to mTNF *in vitro*, I would biotinylate and fluorescently label adalimumab and etanercept and perform binding assays that I would measure by flow cytometry or fluorescent microscopy. Next, I would investigate what, if any, intracellular pathways are activated by the *in vitro* addition of adalimumab and etanercept to cells from patients with RA. In particular, I would examine the p38 pathway and the apoptosis pathways. I would also determine if adalimumab and etanercept treatment of cells from patients with RA differentially activates NFκB, to see if TNF signalling is an important feature of Treg induction.

### 7.12.3 Investigating Treg function in PsA

I think it is necessary to determine if there is in fact a defect in Treg from patients with PsA. To do this, I would examine the capacity of Treg from PsA patients to suppress proliferation and the production of IFNγ and TNFα. I would also repeat suppression assays to determine if etanercept treated patients do 'lose' the capacity to

suppress IL-22 production. Additionally, it would be interesting to see if Treg from patients with PsA can regulate CD8 T cell populations.

If suppression assays confirm that Treg from etanercept treated PsA patients are not capable of suppressing IL-22 and that levels of IL-22 remain high in these patients, I would investigate the inflammatory properties of IL-22 in this setting. In order to dissect the role of IL-22 in PsA it would be preferable to take samples from the skin, joint and blood of patients with PsA. I would first examine IL-22 receptor expression to determine if there is an altered responsiveness to IL-22 in PsA patients treated with etanercept. Then, I think it would be interesting to undertake a approach combining experiments by Sonnenberg et al. [316] and Mitra et al. [410]. I would culture synovial fibroblasts from patients with active PsA with autologous T cells or T cells from etanercept treated patients and measure proliferation of the synovial cells. Then I would differentially block or add-back IL-17 and IL-22 to investigate the capacity of IL-22 to convert from a pro- to anti-inflammatory cytokine in an IL-17-dependent manner.

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# Appendices

## Appendix I. Patient consent form

Royal Free and University College Medical School
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WINDEYER INSTITUTE OF MEDICAL SCIENCES

**DEPARTMENT OF IMMUNOLOGY AND MOLECULAR PATHOLOGY**

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**Project Title:** The function(s) of regulatory T cells in rheumatoid arthritis

**Principal Investigator:** Professor Michael Ehrenstein

**Description of Project:** Investigation into function(s) of regulatory T cells in the above mentioned patients

**Ethical Approval No: 02/0240**

Confidentiality is strictly maintained at all times. Your name will not be known to anyone other than the staff immediately involved in collecting the sample. All data will be anonymous and untraceable to the volunteer except by those immediately involved in collecting the sample.

Please read and sign the following declaration:
I confirm that I have read and understood the information relating to this study. I have had the opportunity to ask questions and I understand that I can withdraw from this study at any time without giving a reason.

**Signature of volunteer:……**
**Print:……..**

**Date:…….**

**Signature of person taking blood:……. Print:**

**Date:…….**